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(71) Applicant (for all designated States except US): SYMBICOM AKTIEBOLAG [SE/SE]; Box 1451, S-901 24 Umeå (SE).

(72) Inventors; and
(75) Inventors/Applicants (for US only) : MARKLUND, Stefan [SE/SE]; Törnskatevägen 26, S-902 37 Umeå (SE). ED-LUND, Thomas [SE/SE]; Tallvägen 56 A, S-902 39 Umeå (SE).

(74) Agent: PLOUGMANN & VINGTOFT; Sankt Annæ Plads 11, DK-1250 Copenhagen K (DK).

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(57) Abstract

Extracellular superoxide dismutase (EC-SOD) variants having the superoxide dismutating property of the native EC-SOD and having a modified (reduced or increased) binding to heparin as compared to recombinant EC-SOD C as well as compositions comprising such variants. The EC-SOD variants are polypeptides comprising: 1) amino acids 1-193 of native EC-SOD C and 2) an amino acid sequence which is based on, but different from amino acid moities 194-222 of recombinant EC-SOD C, either by being truncated or prolonged at the C-terminal end or by having substituted or otherwise modified one or more amino acid moieties of the sequence. Another EC-SOD variant is one which differs from recombinant EC-SOD C by being a glycosylation-free mutant. The variants may be produced by recombinant DNA techniques and are useful in the treatment of various diseases.

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A POLYPEPTIDE

The present invention relates to a polypeptide having the superoxide
dismutating property of native extracellular superoxide dismutase and
a modified, i.e. reduced or increased, affinity for heparin compared
5   to extracellular superoxide dismutase of type C, methods of producing
the polypeptide and the use thereof for the purpose of therapeutic
treatment.

Organisms living in the presence of oxygen have been forced to deve-
lop a number of protective mechanisms against toxic oxygen reduction
10   metabolites, such as superoxide radicals, which are formed in connec-
tion with a variety of biological oxidations. The protective factors
include superoxide dismutases (SOD) (superoxide:superoxide oxido-
reductase, EC 1.15.1.1) which dismutate the superoxide radical and
are found in relatively constant amounts in mammalian cells and
15   tissue. The best known of these enzymes is CuZn SOD which is a dimer
with a molecular weight of 33,000 containing two copper and two zinc
atoms. CuZn SOD is found in the cytosol and in the intermembrane
space of the mitochondria. Mn SOD is a tetramer with a molecular
weight of 85,000 containing 4 Mn atoms, and is mainly located in the
20   mitochondrial matrix. Recently, a superoxide dismutase was found in
the extracellular fluids of mammals, birds and fish. This superoxide
dismutase has been denoted extracellular superoxide dismutase which
in the following will be termed EC-SOD.

EC-SOD is a tetrameric Cu and Zn containing glycoprotein (S. L. Mark-
25   lund, Proc. Natl. Acad. Sci. USA. 79, 1982, pp. 7634-7638; L. Tibell
et al., Proc. Natl. Acad. Sci. USA. 84, 1987, pp. 6634-6638). A cDNA
sequence encoding EC-SOD has been elucidated and useful applications
of the cDNA and the EC-SOD encoded thereby are described in
WO 87/01387, the contents of which are hereby incorporated by refe-
30   rence. The cDNA sequence is the sequence shown in Fig. 1A and Fig. 1B
corresponding to amino acid residues 1-222 (without any mutations).
EC-SOD is a secretory protein and the cDNA encodes an 18 amino acids
long signal sequence which is absent in mature and recombinant EC-SOD
(L. Tibell et al., Proc. Natl. Acad. Sci. USA. 84, 1987, pp. 6634-
35   6638). As deduced from the cDNA sequence, the subunit molecular

weight of the mature enzyme is 24,200. The exact size of the carbohydrate substituent is not known, but the apparent molecular weight on gel chromatography of the tetramer is 140-150 kDa. On SDS-PAGE electrophoresis the subunits display a molecular weight of 30-32 kDa (L.

5   Tibell et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp. 6634-6638). The sequence contains one glycosylation site Asn-89 (K. Hjalmarsson et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp. 6340-6344), and the mature enzyme binds to the lectins concanavalin A, wheat germ lectin and lentil lectin. The tetramers contain 4 Cu and 4 Zn atoms. The

10   active site, which contains the metal atoms, is homologous to the active site of the intracellular CuZn SODs (K. Hjalmarsson et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp. 6340-6344).

EC-SOD isolated from tissues and plasma is heterologous with regard to heparin affinity, and can upon chromatography on Heparin-Sepharose

15   be divided into three subclasses;

    A which does not bind,
    B with intermediate affinity and
    C with relatively strong heparin affinity.

20   *in vivo* the correlate of the heparin affinity is binding to heparan sulfate proteoglycan (K. Karlsson and S. L. Marklund, *Biochem. J. 242*, 1987, pp. 55-59 , K. Karlsson et al., *Biochem. J. 256*, 1988, pp. 29-33; S. L. Marklund and K. Karlsson, *Lab. Invest.* in press, 1989), which occurs on cell surfaces and in the interstitial connective

25   tissue.

In the vasculature, EC-SOD of subclass C, which in the following is termed EC-SOD C, exists in equilibrium between the plasma phase and heparan sulfate proteoglycan in the glycocalyx of the vessel endothelium (K. Karlsson and S. L. Marklund, *Biochem. J. 242*, 1987, pp.

30   55-59; K. Karlsson and S. L. Marklund, *J. Clin. Invest. 82*, 1988, pp. 762-766; K. Karlsson and S. L. Marklund, *Biochem. J. 255*, 1988, pp. 223-228). Upon injection of heparin, bound enzyme is released to plasma because it binds to the heparin instead of the heparan sulfate. This effect is seen only for EC-SOD forms which upon chromato-

35   graphy on Heparin-Sepharose under conditions described in the Analy-

tical Methods section below (Analytical separation of EC-SOD variants
from cell culture media on Heparin-Sepharose) are released by the
NaCl gradient at concentrations above 0.38 M (K. Karlsson and S. L.
Marklund, Biochem. J. 255, 1988, pp. 223-228). Only such forms bind

5   in vivo to vessel endothelium, and with this property the C-class
EC-SODs are defined. B-class EC-SODs (EC-SOD B) show heparin affini-
ty, but are eluted at concentrations below 0.38 M NaCl, and A-class
EC-SODs (EC-SOD A) do not display heparin affinity under the condi-
tions of the Heparin-Sepharose chromatography. EC-SOD Cs appear to

10 bind to the surface of most cell-types in the body, notable excep-
tions are erythrocytes and neutrophil leukocytes. Binding to various
E. coli strains could not be demonstrated (S. L. Marklund and K.
Karlsson, Lab. Invest., Vol. 60, pp 659-666, 1989). This binding
pattern suggests that EC-SOD C has the potential to protect most

15 normal cells in the body, without protecting microorganisms lacking
affinity and without much interfering with superoxide radicals pro-
duced at the surfaces of activated neutrophil leukocytes.

The binding to the strongly negatively charged heparin is apparently
of electrostatic nature (K. Karlsson et al., Biochem. J. 256, 1988,

20 pp. 29-33). The carboxy-terminal end of EC-SOD C carries a strong
positive charge since among the last 20 amino acids 9 are positively
charged. This part of the molecule has been suggested to form the
heparin-receptor structure and Adachi and Marklund (J. Biol. Chem.
264, No. 15, pp. 8537-8541, 1989) suggest that lysine and arginine

25 residues of the C-terminal end are involved in the binding of EC-SOD
C to sulfated glycosaminoglycans (SGAGs), a class of compounds to
which heparin belongs. Thus, Adachi and Marklund have used amino acid
specific reagents in modifying the lysine and arginine residues of
EC-SOD C and obtained enzymes having a reduced affinity to heparin,

30 which enzymes in some cases have lost their superoxide dismutating
activity. Adachi and Marklund did not reveal which lysine and argini-
ne residues of EC-SOD are involved in the heparin binding and did not
disclose the modification of other amino acid residues, and as it
will be understood from the following discussion, the disclosure of

35 Adachi and Marklund does not form part of the present invention.

It may also be noted that the behaviour upon ion exchange chromato-
graphy and isoelectric points of plasma fractions containing EC-SOD A
and ECSOD C has not been established with certainty as being diffe-
rent (Karlsson, K., *Umeå University Medical Dissertations, New Series
5   No 227*, 1988).

In plasma, EC-SOD A and EC-SOD B seem to be quantitatively important,
whereas only minor amounts seem to be present in tissue. Studies up
till now indicate that in the native environment, especially in the
plasma, the three types of EC-SOD are present. In addition, EC-SOD
10   forms an equilibrium between the plasma and endothelial surfaces.

In the treatment of various diseases or disorders it may be advan-
tageous to be able to control the level of circulating EC-SOD in the
plasma and thereby control the degree of plasma and epithelial bind-
ing. Use of EC-SODs of various heparin affinities (reduced or in-
15   creased as compared to EC-SOD C) would be a means of controlling the
circulating level of EC-SOD in the plasma.

EC-SOD C is the only type of EC-SOD which it has been possible to
obtain in large quantities and in a form which is contemplated to be
20   sufficiently pure for therapeutic application, *vide* WO 87/01387 in
which the production of recombinant EC-SOD C is disclosed. EC-SOD A
and EC-SOD B have been observed in plasma and tissue samples in very
low amounts and it has not been possible to isolate these EC-SOD
types in a therapeutically acceptable pure form and in amounts which
25   would make the use thereof in the treatment of various diseases or
disorders realistic.

In order to be able to control the circulating level of EC-SOD in
plasma in connection with treatment of diseases or disorders where
this is advantageous, it is desirable to provide EC-SOD types having
30   various affinities for heparin and thus heparan sulphate present on
cell surfaces, without the superoxide dismutating property thereof
being impaired.

According to the invention, it has been found that the affinity, to
heparin, of polypeptides showing the superoxide dismutating property

of EC-SOD may be modified by suitable modification of the C-terminal amino acid sequence of such polypeptides. A decreased affinity to heparin is obtained when the C-terminal amino acid sequence is trun-cated, and/or when the C-terminal amino acid sequence is conferred with a smaller positive net charge than the net charge of the amino acid sequence constituting amino acid moieties 194-222 of recombinant EC-SOD C.

Thus, in one aspect, the invention relates to a polypeptide having the superoxide dismutating property of the native extracellular superoxide dismutase (EC-SOD) and having a modified binding to hepa-rin as compared with recombinant EC-SOD C,

the polypeptide having the formula

A-R

wherein A designates the EC-SOD C amino acid sequence from and including amino acid moiety No. 1 up to and including amino acid moiety No. 193 (*vide* Fig. 1A or Fig. 1B)

and R is an amino acid sequence conferring to the polypeptide either

a reduced affinity for heparin compared to recombinant EC-SOD C as a result of the sequence

being truncated from its C-terminal end compared to the amino acid sequence constituting amino acid moie-ties 194-222 of recombinant EC-SOD C,

and/or having a smaller positive net charge than the net charge of the amino acid sequence constituting amino acid moieties 194-222 of recombinant EC-SOD C,

or

an increased affinity for heparin compared to recombinant EC-SOD C as a result of the sequence

having a larger positive net charge than the net
charge of the amino acid sequence constituting amino
acid moieties 194-222 of recombinant EC-SOD C,

5   the glycosylation site of the polypeptide in the above
            cases optionally being replaced by a moiety which
            cannot be glycosylated, or

            R is the amino acid sequence constituting amino acid moieties
            194-222 of recombinant EC-SOD C, in which case the glycosylation
10   site of the polypeptide has been replaced with a moiety which
            cannot be glycosylated, whereby the heparin affinity of the
            polypeptide has been increased, compared to recombinant
   EC-SOD C,

     and modifications thereof which retain the superoxide dismutating
15   property of native EC-SOD.

     In the present context, the term "a reduced affinity to heparin"
     indicates that the polypeptide either has no substantial binding to
     heparin under physiological conditions or has a binding to heparin
     which is less strong than the binding of recombinant EC-SOD C, the
20   binding being assessed *in vitro* by elution with NaCl as described in
     the Analytical Method section given below. Thus, the heparin affinity
     is determined by means of the concentration of NaCl required for elu-
     ting the polypeptide when bound to heparin. Correspondingly, the term
     "an increased affinity to heparin" indicates that the polypeptide has
25   a binding to heparin which is stronger than the binding of recombi-
     nant EC-SOD C, the binding again being assessed *in vitro* by elution
     with NaCl.

     As appears from the explanation given below, the heparin affinity of
     the polypeptide is of major importance to the utility of the polypep-
30   tide in connection with various therapeutic, surgical or prophylactic
     treatments, and the present invention now makes it possible to pro-
     vide, in a scale useful for practical use, a range of useful novel
     polypeptides with the superoxide dismutating property of native
     EC-SOD, but with modified heparin affinity.

The term "net charge" indicates the sum of the charges of the amino acid sequence. It is well known that some amino acids, like Arg and Lys, and under certain pH, His, are positively charged, while other amino acids, like Asp and Glu are negatively charged and further

5   amino acids such as glutamine, cysteine, serine, methionine, asparagine, alanine, valine, glycine, leucine isoleucine, proline, threonine, phenylalanine, tyrosine, and tryptophan are neutral or uncharged. To assess the positive net charge of an amino acid sequence, one counts the number of positive charges on amino acids of the se-

10   quence and, from the resulting number, subtracts the number of negative charges. A negative net charge is assessed analogously. The net charge is not to be confused with the charge density, which is calculated by dividing the net charge by the number of amino acids in the sequence.

15   In the present context, "the superoxide dismutating property of native EC-SOD" refers to the capability of the polypeptide (of course, with the appropriate metal association) to catalyze a first-order dismutation of the superoxide radical in a manner corresponding to EC-SOD. EC-SOD and other superoxide dismutases (SODs) catalyzes the

20   following reaction

$$O_2 \cdot^- + O_2 \cdot^- + 2H^+ \quad \rightarrow \quad O_2 + H_2O_2$$
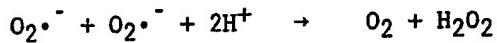
The specific activity of the SODs is very high and is probably mediated by the four Cu atoms of SOD. The rate constant for the catalyzed

25   reaction is about 1×10^9 $M^{-1}s^{-1}$ per Cu atom of the SOD. Because of the superoxide dismutating property of the polypeptide of the invention, the polypeptide is also, in the present context, referred to as a polypeptide which confers or mediates EC-SOD activity. Thus, in the present context the term "EC-SOD activity" is used to indicate the

30   enzymatic or superoxide dismutating activity conferred by the polypeptide of the invention. It should be noted that the term "superoxide dismutating property of native EC-SOD" and related terms should be understood to be qualitative rather than quantitative, that is, relating to the nature rather than the level of activity of the poly-

35   peptide. The superoxide dismutating property of a given polypeptide

may be evaluated by the method set forth in the Analytical Method
section below under the heading: "Analysis of superoxide dismutase
activity". Thus, it will be understood from the above explanation
that "the superoxide dismutating property of native EC-SOD" could

5   also simply be termed "SOD activity" or "SOD enzymatic activity".

The term "native" as used in connection with EC-SOD refers to the
naturally occurring EC-SOD which is found in body tissue or fluid and
which may be any of the naturally occurring EC-SOD types. Native
EC-SOD may e.g. be obtained by extraction from mammalian tissues,

10   e.g. as explained in WO 87/01387.

The term "modifications" is intended to cover proteins or polypep-
tides which have substantially the characteristics of the polypeptide
of the invention outlined above, i.e. the superoxide dismutating
property of EC-SOD and a modified affinity for heparin as compared

15   with recombinant EC-SOD C, but which may, with respect to some amino
acid moieties, be of a composition different from the composition of
the polypeptide of the formula set forth above. Thus, the amino acid
sequence of any part of the polypeptide sequence outlined above may
be modified by amino acid substitution, insertion, deletion, or addi-

20   tion as long as the critical property, i.e. the superoxide dismuta-
ting property, of the polypeptide is not impaired. The modification
may be introduced in the polypeptide moiety R or the polypeptide
moiety corresponding to the part of the EC-SOD amino acid sequence
constituting amino acids 1-193. Furthermore, any modification of the

25   amino acids constituting the polypeptide outlined above, i.e. glyco-
sylation, acetylation, and other possible modifications which do not
impair the superoxide dismutating property of the native EC-SOD is
within the scope of the present invention. Also a subsequence of the
polypeptide outlined above as well as a polypeptide being constituted

30   of 2 or more subsequences should be understood to be within the defi-
nition of a modification of the polypeptide which is within the pre-
sent invention. The substitution, deletion, insertion and/or addition
of amino acid moieties in the polypeptide shown above producing modi-
fications thereof may be obtained in accordance with well known tech-

35   niques, such as site-directed mutagenesis. This will be discussed in
further detail below.

The formula A-R outlined above of the polypeptide of the invention is
constituted of two parts; the first part is the amino acid sequence
of EC-SOD C from and including amino acid 1 and up to and including
amino acid 193. The entire EC-SOD sequence is constituted of 222

5   amino acids, not all of which are required for conferring the super-
oxide dismutating property to native EC-SOD C. Thus, by comparison of
the amino acid sequence with Cu Zn SODs of various origins, a pos-
sible active site of the enzyme has been found between amino acid 97
and amino acid 193 of the amino acid sequence encoding EC-SOD C. This

10   part of EC-SOD C is believed to be essential for the superoxide dis-
n. ating property of the enzyme. The part of the EC-SOD C constitu-
ı. ıg amino acid 1-96 is contemplated to be involved in the formation
of oligomers of the polypeptide. However, in accordance with what is
stated above, modifications within the part of the EC-SOD C sequence

15   constituting amino acids 1-193, which modifications do not impair the
superoxide dismutating property of the enzyme should be considered as
modifications of the polypeptide of the invention in accordance with
the definition given above. Based on the above-mentioned homology
between the active sites of the various SOD enzymes, modifications in

20   the part of the EC-SOD C sequence constituting amino acids 1-193
could be performed by site-directed mutagenesis so that the active
site of the enzyme remains active. How to perform such modifications
is explained in further details below. The amino acid sequence R of
the polypeptide of the invention is the part of the polypeptide which

25   has been found, according to the invention, to be relevant with
respect to modifications of the heparin affinity of the enzyme.
Normally, R is based on the amino acids moieties 194-222 of recombi-
nant EC-SOD C, i.e. the amino acid moieties of the C-terminal part of
EC-SOD C, R, however, having been changed in accordance with the

30   present invention in such a manner that a reduced or an increased
affinity for heparin has been obtained compared to the heparin af-
finity of recombinant EC-SOD C. The changes of the C-terminal part of
EC-SOD C which produce the amino acid sequence R will be further
dealt with in the following. In adddition to such changes, R may in

35   some cases have been subjected to modifications as defined above,
e.g. modifications in R which are not involved in the heparin bin-
ding.

Because of the high degree of homology between the polypeptide of the invention and EC-SOD, the polypeptide of the invention is also refer-red to as a EC-SOD variant.

The modified affinity for heparin of the polypeptide of the invention
5   as compared with the binding to heparin of recombinant EC-SOD C may
be either a reduced or an increased affinity. An increased affinity
may be advantageous when it is desirable to provide a strong binding
of the polypeptide to cell surfaces, e.g. in connection with trans-
plantation of organs or when the EC-SOD activity is injected directly
10   into an inflamed organ where it is to bind. A reduced affinity for
heparin may be advantageous when it is desirable to provide a high
amount of EC-SOD activity circulating in the body fluid so as to
ensure that freely circulating EC-SOD variants is available for
sufficient time after injection to move to the tissue or cell where
15   it is needed without immediately being bound to vascular cell sur-
faces when brought in contact therewith.

The term "the glycosylation site" means the Asn moiety of position 89
of the polypeptide and - where appropriate - any other moiety of the
polypeptide which can be glycosylated. (In the unmodified polypep-
20   tide, the Asn moiety of position 89 is the only moiety which can be
glycosylated).

It has surprisingly been found (*vide* Example 4) that substituting the
glycosylation site of EC-SOD C with a non-glycosylation site results
in a polypeptide having increased affinity for heparin compared to
25   recombinant EC-SOD C. It is surprising that a glycosylation-free
polypeptide has a marked increased heparin affinity compared to re-
combinant EC-SOD C because the glycosylation site of EC-SOD C, the
sequence of which appears from Fig. 1A and 1B, is found at position
89 of the sequence, i.e. in the N-terminal part of the polypeptide
30   which would not be believed to be of importance for heparin binding.
A glycosylation-free polypeptide has the advantage that it may be
produced in an organism which is not capable of carrying out glycosy-
lation. Thus, e.g., a glycosylation-free polypeptide may be produced
in *E. coli* which is a well known and widely used host organism for

the production of recombinant polypeptides. The production in *E. coli*
is normally very simple and does not require that any extensive
measures are taken as opposed to the use of various cell lines as
host organisms in the recombinant production of polypeptides, which

5   cell lines should be handled more carefully. The expenses of recombi-
nant production in *E. coli* are lower than the expenses of the recom-
binant production in cell lines derived from higher organisms.

Preferably, the smaller positive net charge of R compared to the net
charge of the amino acid sequence constituting amino acid moieties

10   194-222 of recombinant EC-SOD C has been obtained by

removal of one or several positively charged amino acid moieties
compared to the amino acid sequence constituting amino acid
moieties 194-222 of recombinant EC-SOD C

and/or substitution of one or several positively charged amino

15   acid moieties of the amino acid sequence constituting amino acid
moieties 194-222 of recombinant EC-SOD C with negatively charged
and/or neutral amino acid moieties,

and/or addition of negatively charged amino acid moieties,
compared to the corresponding sequence in EC-SOD C,

20   and/or chemical modification of one or more amino acid moieties
to introduce negative charges.

The methods by which these various modifications of the amino acid
sequence are carried out is described in further detail below.

In one embodiment, R designates a truncated version of the amino acid

25   sequence constituting amino acid moieties 194-222 of recombinant
EC-SOD C, the C-terminal end of the sequence having been truncated
with 1-12 amino acids. The truncation may be accomplished by use of
site-directed mutagenesis as described below. As it is described in
the examples, truncation of different numbers of amino acids results

30   in polypeptides having different affinities for heparin. Thus, trun-
cation of 6 amino acids results in a polypeptide having a slightly

reduced affinity for heparin (variant T216 described above), whereas
truncation of 7 amino acids surprisingly results in a polypeptide
having substantially no affinity for heparin (variant T215), i.e. a
polypeptide of EC-SOD A type. Thus, the presence of an amino acid in
5   position 216 seems to be important for conferring to the polypeptide
an affinity for heparin; the presence of a cluster of positively
charged amino acids (amino acids 210-214) is surprisingly not in
itself sufficient for ensuring binding to heparin. Thus, when a
reduced but not totally lacking heparin affinity is desirable, it is
10   preferred that the amino acid sequence R comprises at least 23 amino
acid moieties.

In variant T216, position 216 is occupied by the negatively charged
glutamine, but other experiments (vide Example 4) have indicated that
the charge of the amino acid in this position is of minor importance
15   to the heparin affinity of the polypeptide. Thus, in addition to net
charge, the structure of the polypeptide is contemplated to be impor-
tant for binding to heparin. Accordingly, in a preferred embodiment,
the C-terminal end has been truncated with 1-6 amino acids, such as
with 1-5 amino acids, which in most cases will ensure some binding to
20   heparin as will be explained in further detail below.

In another preferred embodiment, the larger positive net charge of R
compared to the net charge of the amino acid sequence constituting
amino acid moieties 194-222 of recombinant EC-SOD C has been obtained
by

25   removal of negatively charged amino acid moieties compared to
            the amino acid sequence constituting amino acid moieties 194-222
            of recombinant EC-SOD C,

            and/or substitution of neutral and/or negatively charged amino
            acid moieties with positively charged amino acid moieties,
30   compared to the amino acid sequence constituting amino acid
            moieties 194-222 of recombinant EC-SOD C,

            and/or addition of positively charged amino acid moieties,

compared to the amino acid sequence constituting amino acid
moieties 194-222 of recombinant EC-SOD C,

and/or chemical modification of one or more amino acid moieties
to introduce positive charges,

5   which produces a polypeptide having an increased affinity for
heparin.

Preferably, the amino acid sequence of the polypeptide of the inven-
tion contains one or several of the following amino acid moieties:
Arg, Lys, Glu, Ser, Cys, and His. The presence of these amino acid

10 moieties in R will normally give rise to a modified affinity for
heparin compared to recombinant EC-SOD C.

One example of a polypeptide of the invention is the polypeptide T216
referred to above, which polypeptide has a reduced binding to heparin

15 as compared with recombinant EC-SOD C. The amino acid sequence R in
the polypeptide T216 is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-
Arg-Glu-His-Ser-Glu-Arg-Lys-Lys-Arg-Arg-Arg-Glu. The polypeptide T216
has a slightly reduced affinity for heparin compared to EC-SOD C and
has been shown to have the superoxide dismutating property of native

20 EC-SOD. The polypeptide T216 is further described in Example 1, 2 and
4 below.

Another example of a polypeptide of the invention is the polypeptide
termed T215 which has a reduced binding to heparin as compared with
recombinant EC-SOD C. In the polypeptide T215, R is Val-Cys-Gly-Pro-

25 Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-Arg-Lys-Lys-Arg-Arg-
Arg. The polypeptide T215 is further described in Examples 1, 2 and 4
below.

Still another example of a polypeptide of the invention is the poly-
peptide termed T213 which has a reduced binding to heparin as com-

30 pared with recombinant EC-SOD C. In the polypeptide T213, R is Val-
Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-Arg-Lys-
Lys-Arg. The polypeptide T213 is further described in Examples 1, 2
and 4 below.

A further example of a polypeptide of the invention is the polypep-
tide termed T209 which has a reduced binding to heparin as compared
with recombinant EC-SOD C. In the polypeptide T209, R is Val-Cys-Gly-
Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu. The polypeptide
5   T209 is further described in Examples 1, 2 and 4 below.

An example of a polypeptide of the invention having an increased
binding to heparin as compared with recombinant EC-SOD C and which is
non-glycosylated, is the polypeptide G1, wherein the glycosylation
site Asn 89 in the polypeptide of native EC-SOD C has been replaced
10   with a Gln-moiety. The polypeptide G1 is further described in
Examples 1, 2 and 4 below.

Still another example of a polypeptide of the invention having a
reduced binding to heparin as compared with recombinant EC-SOD C is
the polypeptide SA220. The amino acid sequence R in the polypeptide
15   SA220 is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-
Glu-Arg-Lys-Lys-Arg-Arg-Arg-Glu-Ser-Glu-Cys-Ala-Ala-Ala wherein the
amino acid Lys 220 of the native EC-SOD C has been replaced with an
Ala moiety. The polypeptide SA220 is further described in Examples 1,
2 and 4 below.

20   An example of a polypeptide of the invention having an increased
binding to heparin as compared with recombinant EC-SOD C is the poly-
peptide SA216. The amino acid sequence R in the polypeptide SA216 is
Val-Cys-Gly-Pro-Gly-Leu-trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-Arg-
Lys-Lys-Arg-Arg-Arg-Ala-Ser-Glu-Cys-Lys-Ala-Ala wherein the amino
25   acid Glu 216 of the native EC-SOD C has been replaced with an Ala
moiety. The polypeptide SA216 is further described in Examples 1, 2
and 4 below.

Still another example of a polypeptide of the invention having a
reduced binding to heparin as compared with recombinant EC-SOD C is
30   the polypeptide SAT216. The amino acid sequence R in the polypeptide
SAT216 is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-
Ser-Glu-Arg-Lys-Lys-Arg-Arg-Arg-Ala wherein the amino acid Glu 216 of

the native EC-SOD C has been replaced with an Ala moiety and the rest of the C-terminal end has been truncated.

The polypeptide SA219 may be used as a reference example illustrating that the substitution of a neutral amino acid with another

5 neutral amino acid in position 219 results in a polypeptide having the same binding to heparin as recombinant EC-SOD C. Thus, when the net charge remains un· ·anged, no modification in heparin binding is observed. The amino a· d sequence R in the polypeptide SA219 is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-Arg-Lys-

10 Lys-Arg-Arg-Arg-Glu-Ser-Glu-Ala-Lys-Ala-Ala wherein the neutral amino acid Cys 219 of the native EC-SOD C has been replaced with another neutral amino acid, namely an Ala moiety. The polypeptide SA219 is further described in Examples 1, 2 and 4 below.

The polypeptide of the invention having the superoxide dismutating

15 property of native EC-SOD and a modified affinity for heparin as compared with recombinant EC-SOD C is preferable of recombinant origin. In the present context, the term "recombinant" is intended to indicate that the polypeptide is derived from a cell which has been subjected to recombinant DNA techniques, i.e. into which a DNA se-

20 quence coding for the polypeptide has been inserted and which has subsequently been induced to express the polypeptide. However, as it will be explained below the polypeptide of the invention may also be obtained from EC-SOD which is produced by a cell line, e.g. of human origin, and which subsequent to its production is modified, e.g. che-

25 mically or enzymatically, to result in the polypeptide of the invention.

In accordance herewith, a further and important aspect of the present invention is a polypeptide composition which has the superoxide dis-mutating property of native EC-SOD and which comprises several of the

30 above defined polypeptides of the invention, or one or more of the polypeptides of the invention in admixture with EC-SOD A, EC-SOD B or EC-SOD C.

The polypeptide(s) of the invention from which the polypeptide com-position is formed may be one(s) which either has/have a reduced or

an increased affinity for heparin compared to recombinant EC-SOD C.
The polypeptide(s) of the invention may be the only constituent(s) of
the polypeptide composition. This is advantageous because the poly-
peptides may be produced rather easily by recombinant techniques,

5   e.g. as described below, and may be constructed so as to provide a
polypeptide composition having a specific desirable affinity for
heparin (which may be different from the affinity defined by types A,
and B and C). However, the polypeptide(s) of the invention may also
be used in admixture with EC-SOD A, EC-SOD B or EC-SOD C which may

10   either be native or produced in accordance with the principles of the
present invention, e.g. as described below. EC-SOD C is easily avail-
able in a recombinant form, which also, by means of the present in-
vention applies for EC-SOD A and EC-SOD B. Admixing one or more of
the polypeptides of the invention with EC-SOD A, EC-SOD B and/or

15   EC-SOD C may be advantageous in order to confer to the polypeptide
composition a specific and desirable affinity for heparin.

Thus, in another aspect, the polypeptide composition which has the
superoxide dismutating property of native EC-SOD is an oligomer which
comprises several polypeptides of the invention, or one or more of

20   the polypeptides in admixture with EC-SOD A, EC-SOD B or EC-SOD C.
The oligomer is presumed to be formed when the polypeptides of the
invention or the polypeptide(s) and the EC-SOD A, EC-SOD B or EC-SOD
C are contacted with each other, e.g. in a suitable solution similar-
ly to what is believed to happen in vivo where the tetrameric EC-SOD

25   is automatically formed.

Preferably, the polypeptide composition of the invention is in the
form of a tetramer comprising four polypeptides as defined above, or
one or more of the polypeptides in admixture with EC-SOD A, EC-SOD B
or EC-SOD C.

30   The polypeptides of the polypeptide composition of the invention may
either be identical or different.

The polypeptide composition of the invention described above may have
a lower mean heparin binding than recombinant EC-SOD C. The term
"mean heparin binding" is used because the different polypeptide

constituents of the polypeptide composition may have different
affinities for heparin and thus be bound to a greater or smaller
extent to heparin or heparan sulphate at cell surfaces. However, the
mean heparin binding is the overall binding of the composition.

5   Examples of various polypeptide compositions of the invention are:

        a polypeptide composition which is composed of two polypeptides
        having reduced affinity for heparin compared to recombinant
        EC-SOD C, for instance, of the polypeptide termed T216 and the
        polypeptide T213 described herein, or

10   a polypeptide composition which is composed of a polypeptide
        having reduced heparin affinity compared to recombinant
        EC-SOD C, e.g. the polypeptide termed T216 described herein, and
        recombinant EC-SOD C,

     The polypeptide or polypeptide composition of the invention may be
15   one which has an increased affinity for heparin compared to recom-
     binant EC-SOD C and which has a (mean where appropriate) heparin
     binding affinity corresponding to complete elution of the polypeptide
     or polypeptide composition from a heparin-Sepharose column with
     between 0.55 and 1.5 molar aqueous NaCl under conditions as descri-
20   bed in the Analytical Methods section herein under the heading "Sepa-
     ration of EC-SOD variants from cell culture media by Heparin-Sepharo-
     se Chromatography". Elution of the polypeptide or polypeptide compo-
     sition with a molar aqueous NaCl of a concentration of about 1.5 re-
     flects a very strong affinity for heparin, whereas elution with NaCl
25   of a concentration of 0.55 M reflects an affinity for heparin which
     is very close to the affinity of recombinant EC-SOD C. Preferably,
     the polypeptide or polypeptide composition of the invention has a
     (mean where appropriate) heparin binding affinity corresponding to
     complete elution of the polypeptide or polypeptide composition from a
30   heparin-Sepharose column with between 0.55 and 1.0 molar aqueous NaCl
     or 0.65-1.0 M NaCl or, such as with between 0.55 and 0.75 molar
     aqueous NaCl or 0.60-1.0 M NaCl, under conditions as described in the
     Analytical Methods section herein under the heading "Separation of
     EC-SOD variants from cell culture media by Heparin-Sepharose Chroma-

tography". More preferably, the polypeptide or polypeptide composition according of the invention has a (mean where appropriate) heparin binding affinity corresponding to complete elution of the polypeptide or polypeptide composition from a heparin-Sepharose column of

5 between 0.55 and 0.65 molar aqueous NaCl under conditions as described in the Analytical Methods section herein under the heading "Separation of EC-SOD variants from cell culture media by Heparin-Sepharose Chromatography".

A special embodiment of the invention relates to polypeptides or

10 polypeptide compositions of the type described above having a very substantially increased heparin affinity compared to recombinant EC-SOD C, corresponding to complete elution of the polypeptide or polypeptide composition from a heparin-Sepharose column, but an aqueous NaCl concentration which are above 1.5 under the conditions

15 described in the Analytical Methods section. Such embodiments showing extremely strong heparin binding are also within the scope of the present invention.

A polypeptide or polypeptide composition of the invention which has a reduced affinity for heparin compared to recombinant EC-SOD C nor-

20 mally has a (mean where appropriate) heparin binding affinity corresponding to complete elution of the polypeptide or polypeptide composition from a heparin-Sepharose column with between 0 and 0.54 molar aqueous NaCl under conditions as described in the Analytical Methods section herein under the heading "Separation of EC-SOD variants from

25 cell culture media by Heparin-Sepharose Chromatography". Elution of the polypeptide or polypeptide composition with NaCl of a concentration close to 0 M reflects an A-type affinity for heparin, whereas elution with 0.54 M reflects a slightly reduced C-type. Preferably, the polypeptide or polypeptide composition of the invention has a

30 (mean where appropriate) heparin binding affinity corresponding to complete elution of the polypeptide or polypeptide composition from a heparin-Sepharose column with between 0.0 and 0.50 molar aqueous NaCl, such as between 0.0 and 0.45 molar aqueous NaCl, e.g. between 0.0 and 0.35 molar aqueous NaCl, such as 0.10-0.25 molar aqueous

35 NaCl, under conditions as described in the Analytical Methods section

herein under the heading "Separation of EC-SOD variants from cell
culture media by Heparin-Sepharose Chromatography".

As explained above, the polypeptide of the invention is normally a
highly complex molecule which in most cases is glycosylated. Normal-
5   ly, the glycosylation site is the asparagine moiety at position 89
of the amino acid sequence constituting the polypeptide, which aspa-
ragine moiety is also the glycosylation site of native EC-SOD. In
accordance with the present invention, polypeptides are contemplated
which have one or more glycosylation sites different from the aspara-
10 gine 89 and which have the superoxide dismutating property of native
EC-SOD and a modified affinity for heparin compared to recombinant
EC-SOD C. Such polypeptides are also to be understood to be within
the present invention.

Alternatively, as it is explained above it has been possible, in
15 accordance with the principles of the present invention, to construct
and produce a non-glycosylated polypeptide which lacks the glycosyla-
tion site asparagine 89, which polypeptide is termed G1. This poly-
peptide has been found to have the superoxide dismutating property of
native EC-SOD and an increased affinity for heparin compared to re-
20 combinant EC-SOD C. Also other non-glycosylated polypeptides are en-
visaged, which polypeptides have the superoxide dismutating property
of recombinant EC-SOD and a modified, increased or reduced, affinity
for heparin compared to recombinant EC-SOD C. Thus, for instance such
a non-glycosylated polypeptide may be obtained on the basis of the
25 polypeptide G1 by modifying this polypeptide by a suitable method,
e.g. site-directed mutagenesis, as described in further details be-
low. Preferably such modifications are performed in the C-terminal
part of G1. Of course, non-glycosylated polypeptides having the
superoxide dismutating property of native EC-SOD and a modified affi-
30 nity for heparin as compared to recombinant EC-SOD C may be construc-
ted independently of the polypeptide G1, e.g. by substituting the
glycosylation site asparagine 89 of the amino acid sequence of the
polypeptide of the invention with an amino acid different from gluta-
mine, the amino acid used in the polypeptide G1, and if desirable
35 otherwise modify the sequence. Any non-glycosylated polypeptide which
has the critical properties of the polypeptide of the invention, i.e.

the superoxide dismutating property of native EC-SOD and a modified
affinity for heparin compared to recombinant EC-SOD C, is to be
understood as being within the scope of the present invention.

Furthermore, the present invention relates to a DNA sequence encoding
5   a polypeptide as defined above or modifications thereof having the
superoxide dismutating property of recombinant EC-SOD and having a
modified binding to heparin as compared with recombinant EC-SOD C.
Examples of a DNA sequence according to the invention are the sequen-
ces shown in Fig. 1A, Fig. 1B and Fig. 1C, or a modification thereof
10 which encodes a polypeptide having the superoxide dismutating pro-
perty of native EC-SOD and showing a modified heparin binding affi-
nity as a result of one of the modifications mentioned above. The DNA
sequence may be of synthetic origin, i.e. prepared synthetically by
established standard methods, e.g. as described by Matthes et al.,
15 *EMBO Journal 3*, 1984, pp. 801-805. Alternatively, a DNA fragment
encoding a polypeptide of the invention or a modification thereof
which has a superoxide dismutating property of recombinant EC-SOD may
be obtained by modification of a DNA sequence of complementary DNA
(cDNA) origin or of genomic origin, which DNA sequence encodes native
20 EC-SOD C. This will be explained in further detail below.

In a further aspect, the present invention relates to a replicable
expression vector which comprises a DNA sequence encoding EC-SOD. In
the present context, the term "replicable" means that the vector is
able to replicate in a given type of host cell into which it has been
25 introduced. The vector may be one carrying the DNA sequence shown
above or any suitable modification thereof as explained above. Imme-
diately upstream of this sequence (the coding sequence of EC-SOD)
there may be provided a sequence coding for a signal peptide, the
presence of which ensures secretion of the EC-SOD expressed by host
30 cells harbouring the vector. The signal sequence may, for instance,
be the following sequence:

```
        -18                     -10                        -1
MetLeuAlaLeuLeuCysSerCysLeuLeuLeuAlaAlaGlyAlaSerAspAla
ATGCTGGCGCTACTGTGTTCCTGCCTGCTCCTGGCAGCCGGTGCCTCGGACGCC
TACGACCGCGATGACACAAGGACGGACGAGGACCGTCGGCCACGGAGCCTGCGG
                                                      120
```

which sequence shows the signal sequence and signal peptide preceding
the sequence encoding EC-SOD C.

The vector may be any vector which may conveniently be subjected to
recombinant DNA procedures, and the choice of vector will often
depend on the host cell into which it is to be introduced. Thus, the
vector may be an autonomously replicating vector, i.e. a vector which
exists as an extrachromosomal entity, the replication of which is
independent of chromosomal replication; examples of such a vector are
a plasmid, phage, cosmid, mini-chromosome or virus. Alternatively,
the vector may be one which, when introduced in a host cell, is
integrated in the host cell genome and replicated together with the
chromosome(s) into which it has been integrated.

The present invention further relates to a cell harbouring a repli-
cable expression vector as defined above. In principle, this cell may
be of any type of cell, i.e. a prokaryotic cell such as a bacterium,
e.g. *E. coli*, a unicellular eukaryotic organism, a fungus or yeast,
or a cell derived from a multicellular organism, e.g. an animal or a
plant. It is, however, believed that a mammalian cell may be particu-
larly capable of expressing a polypeptide of the invention which in
most aspects is a rather complex glycosylated molecule which cells of
lower organisms might not be able to produce. Examples of such cells
are CHO-DXB 11/pPEE6-pSV2Ddhfr deposited on September 14, 1989, in
the European Collection of Animal Cell Cultures under the Accession
Number ECACC 89091402 and CHO-DXP '1/pPEE7-pSV2dhfr deposited on
September 14, 1989, in the Europ   Collection of Animal Cell Cul-
tures under the Accession Number ₥ACC 89091403, which cells have
been deposited under the provision of the Budapest Treaty. *E. coli*
may advantageously be used for the production of EC-SOD variants
according to the invention, which variants are less complex molecules

such as, e.g., a glycosylation-free variant (e.g. the variant G1
described herein).

In a further aspect the present invention relates to a method of
producing a polypeptide as defined above having a modified affinity

5   for heparin compared to recombinant EC-SOD C and having the super-
oxide dismutating property of native EC-SOD which method comprises
modifying a DNA sequence encoding native EC-SOD C by a mutagenic
treatment so as to obtain a DNA fragment as defined above encoding
the polypeptide, inserting said DNA fragment into a vector which is

10   able to replicate in a host cell, introducing the resulting recom-
binant vector into the host cell, culturing the host cell in an
appropriate culture medium under appropriate conditions for expres-
sing the polypeptide, and recovering the polypeptide from the host
cell or culture medium.

15   The medium used to grow the cells may be any conventional medium
suitable for the purpose. Because of the high degree of similarity of
the polypeptide of the invention and EC-SOD, the polypeptide of the
invention is believed to contain Cu and Zn and it may be necessary to
add extra Cu and/or Zn for the synthesis of the polypeptide of the

20   invention, especially if the polypeptide is to be produced in in-
creased amounts. A suitable vector may be any of the vectors descri-
bed above, and an appropriate host cell may be any of the cell types
listed above. The methods employed to construct the vector and effect
introduction thereof into the host cell may be any methods known for

25   such purposes within the field of recombinant DNA.

The DNA sequence to be modified may be of cDNA or genomic origin as
discussed above, but may also be of synthetic origin. Furthermore,
the DNA sequence may be of mixed cDNA and genomic, mixed cDNA and
synthetic or genomic and synthetic origin as discussed above. While

30   the present discussion deals primarily with modifications which use a
DNA sequence encoding native EC-SOD C as the DNA starting material,
it is evident that because the amino acid sequence of EC-SOD C and
the nucleotide sequence of DNA encoding native EC-SOD C are known,
the DNA starting material could also be a DNA sequence which does not

35   encode native EC-SOD C but rather a modification thereof, such DNA

sequence then being modified, e.g. by site-directed mutagenesis, to
result in the desired DNA fragment encoding the desired EC-SOD vari-
ant. The following discussion focused around modifications of DNA
encoding native EC-SOD C should be understood to encompass also such
5   possibilities, as well as the possibility of building up the DNA by
ligation of two or more DNA fragments to obtain the desired DNA
fragment, and combinations of the above-mentioned principles.

The DNA sequence, e.g. a DNA sequence encoding native EC-SOD, may be
modified using any suitable technique which results in the production
10   of a DNA fragment encoding a polypeptide of the invention. The DNA
sequence may be modified in the sequence encoding part A of the
polypeptide of the invention, i.e. amino acids 1-193 of EC-SOD C, or
in the sequence including the amino acid sequence R of the polypepti-
de of the invention.

15   The modification of the DNA sequence encoding the amino acid sequence
A of the polypeptide of the invention should be one which does not
impair the superoxide dismutating property of the resulting polypep-
tide. Such modification may be performed on the basis of a comparison
between the amino acid sequence of the polypeptide of the invention
20   and amino acid sequences of other SOD enzymes (the great homology
between the active site of EC-SOD and of other human SOD enzymes as
well as of SOD enzymes from widely varying non-human sources is known
and described, e.g., in Hjalmarsson et al., *Proc. Natl. Acad. Sci.
USA 84*, 1987, pp. 6340-6344). On the basis of the comparison, the
25   amino acids of the polypeptide of the invention which are most likely
to be involved in its superoxide dismutating activity may be determi-
ned and modifications may be performed either in a region which is
not contemplated to form the active site, or by modifying codons in
the active site region which encode unconserved amino acid residues
30   (on the basis of the amino acid comparison with other SOD enzymes).
Furthermore, nucleotide substitutions may be made which substitutions
result in a translation into the same amino acid (i.e. having either
exchanged one codon with another codon encoding the same amino acid,
or having exchanged a nucleotide of a codon, which nucleotide is not
35   essential for the translation into the given amino acid).

Similarly, modification in the DNA sequence encoding part R of the polypeptide of the invention may be performed, which modification is one which either results in a modification of the heparin binding affinity of the resulting polypeptide or one which does not change

5   the heparin binding affinity. In the latter case, one or more nucleotide substitutions may be performed which substitutions either lead to the substitution of one amino acid for another, which amino acid is not involved in the heparin binding, or which result in a changed codon which is translated into the same amino acid.

10   One method which has been found to be of particular use in modifying DNA sequences is the above-mentioned site-directed mutagenesis, but of course, random mutations in the DNA sequence encoding the polypeptide of the invention may also result in modified DNA sequences as described above.

15   Site-directed mutagenesis is a well known and very specific technique for specifically changing (mutating) nucleotides of a given nucleotide sequence, which technique is also termed oligonucleotide-directed mutagenesis. The basic principle of site-directed mutagenesis is to prime in-vitro DNA synthesis with an oligonucleotide, which is

20   conveniently chemically synthesized, which oligonucleotide to a large extent matches the DNA sequence to be modified. The position(s) of the oligonucleotide corresponding to the position(s) of the DNA sequence to be changed do/does, however, not match, and the mismatching nucleotide(s) will be introduced into the DNA sequence upon replica-

25   tion. Preferably the site-specific mutagenesis is carried out on a single strand of the DNA sequence encoding EC-SOD C by use of an oligonucleotide which is identical to a subsequence of one of the strands of the DNA sequence encoding EC-SOD C except for at least one nucleotide which has been inserted, deleted, substituted or added to

30   the sequence.

Conveniently, the single stranded DNA is obtained by cloning in M13 and the oligonucleotide is annealed to the single stranded DNA present in M13 which is subsequently replicated and transformed into a suitable host organism such as *E. coli*. The resulting mutants may be

found by screening the *E. coli* clones with the oligonucleotide pro-
vided with a suitable label such as e.g. ^{32}P.

Normally, the oligonucleotide which is used for the site-directed
mutagenesis to produce the polypeptide of the invention differs from
5   the corresponding part of the DNA sequence encoding EC-SOD C in that

a) it has an insert within its sequence of at least 3 nucleo-
tides such as at least 6 nucleotides which is different from the
corresponding part of the DNA sequence encoding EC-SOD C,

b) its sequence is at least 3 nucleotides, such as at least 6
10   nucleotides shorter than the corresponding part of the DNA
sequence encoding EC-SOD C because of deletion of these nucleo-
tides within or at one or both of the ends of the sequence,

c) its sequence differs from the corresponding part of the DNA
15   sequence encoding EC-SOD C in that at least one nucleotide of
the DNA sequence encoding EC-SOD C has been substituted with
another nucleotide, and/or

d) at least one nucleotide has been added to one or both of the
ends of the DNA sequence encoding EC-SOD C.

20   Normally, the oligonucleotide used comprises 5-150 nucleotides, pre-
ferably 5-50, more preferably 7-30, still more preferably 7-25 and
most preferable about 21 or 23 nucleotides.

For the production of a polypeptide having the superoxide dismutating
property of native EC-SOD and a reduced affinity for heparin as com-
25   pared to recombinant EC-SOD C, for instance, one or several of the
following strategies may be used, either separately or in combina-
tion:

a) introducing a stop codon in the C-terminal part of the DNA
sequence encoding EC-SOD C by site-directed mutagenesis as
30   described above using an oligonucleotide which is substantially
identical to the 3'-part of the DNA sequence encoding native

EC-SOD C except for the presence of a stop codon in the 3'-end
of the oligonucleotide, so as to obtain a modified DNA sequence
which results in the expression of a polypeptide which is trun-
cated from its C-terminal end by one or more amino acid moieties

5   and which has a reduced affinity for heparin compared to recom-
binant EC-SOD C. The stop codons may be introduced upstreams for
one or more codons of the C-terminal end of EC-SOD C encoding
positively charged amino acid moieties, e.g. Lys and/or Arg, so
that a polypeptide is obtained which is truncated by one or more

10   positively charged amino acids moieties. The truncation may
alternatively be performed by an enzymatic treatment using
enzymes which specifically cleaves or otherwise modifies the
polypeptide sequence at specific amino acids moieties or by a
chemical treatment.

15   b) Substituting one or more codons of the C-terminal part of the
DNA sequence encoding EC-SOD C which codon(s) encode(s) posi-
tively charged amino acids such as Lys and/or Arg, with codons
encoding neutral and/or negatively charged amino acid by site-
directed mutagenesis using an oligonucleotide being complemen-

20   tary to one strand of at least a part of the C-terminal part of
the DNA sequence, except for nucleotides of the codons to be
substituted.

c) Adding one or more codons encoding negatively charged amino
acid moieties such as Asp and/or Glu to the C-terminal part of

25   the DNA sequence encoding EC-SOD C, so as to obtain a modified
DNA sequence encoding a polypeptide having added one or more
negatively charged amino acid moieties in its C-terminal part
by use of site-directed mutagenesis using an oligonucleotide
corresponding to the C-terminal part except for nucleotides of

30   the codon(s) to be substituted; or by adding negatively charged
amino acids to the amino acid sequence of EC-SOD C by peptide
synthesis in accordance with well known techniques; or by adding
codon(s) encoding negatively charged amino acids such as Asp
and/or Glu to the DNA sequence encoding EC-SOD C by nucleotide

35   synthesis.

d) Introducing negatively charged groups in the amino acid
moieties of EC-SOD C by chemical synthesis in accordance with
well-known methods. Thus, it may be possible to modify groups
present in the amino acid moieties of the polypeptide by chang-

5   ing, e.g. the -SH group in Cys to the corresponding sulphonic
acid group and the -OH group in Ser to the corresponding car-
boxylic acid group, or to introduce new groups into the amino
acid moieties by using the nucleophilic property of -SH and -NH
in Cys and Lys, respectively and possibly Arg, by reacting with

10   a reagent containing a leaving group and a carboxylic acid group
or a sulphonic acid group.

The truncation may alternatively be performed by an enzymatic treat-
ment using enzymes which specifically cleaves or otherwise modifies
the polypeptide seque.ice at specific amino acids moieties. Specific

15 amino acid moieties may be modified by use of amino acid specific
reagents, provided that these reagents modify only amino acid moie-
ties which form part of the active site of the polypeptide.

For the production of a polypeptide having the superoxide dismutating
property of native EC-SOD and an increased affinity for heparin as

20 compared to recombinant EC-SOD C, for instance, one or several of the
following strategies may be used either alone or in combination:

a) Substituting one or more codons of the C-terminal part of the
DNA sequence encoding EC-SOD C which codon(s) encode(s) neutral
and/or negatively charged amino acids, e.g. Ser, Glu, Cys,

25   and/or Ala, with codons encoding positively charged amino acid
moieties, e.g. Arg, His and/or Lys, by site-directed mutage-
nesis using an oligonucleotide being complementary to one strand
of at least a part of the C-terminal part of the DNA sequence,
except for nucleotides of the codons to be substituted.

30   c) Adding one or more codons encoding positively charged amino
acid moieties, e.g. Arg, His and/or Lys, to the C-terminal part
of the DNA sequence encoding EC-SOD C, so as to obtain a modi-
fied DNA sequence encoding a polypeptide having added one or
more positively charged amino acid moieties in its C-terminal

part by use of site-directed mutagenesis using an oligonucleo-
tide corresponding to the C-terminal part except for nucleotides
of the codon(s) to be substituted; or by adding positively
charged amino acids to the amino acid sequence of EC-SOD C by

5   peptide synthesis in accordance with well known techniques; or
by adding codon(s) encoding positively charged amino acids to
the DNA sequence encoding EC-SOD C by nucleotide synthesis.

d) Introducing positively charged groups in the amino acid
moieties of EC-SOD C by chemical synthesis in accordance with

10   well known-methods. Thus it may be possible to modify groups
present in the amino acid moieties of the polypeptide by, e.g.,
quarternisation of N-atoms in the side chain, in particular in
Lys, Arg and His, or by introducing new groups using the nucleo-
philic property of -SH and -NH in Cys and Lys, respectively, and

15   possibly, Arg, by reacting with a reagent containing a leaving
group and an amino function followed by quarternisation of the
amino function.

Specific examples of site-directed mutagenesis carried out in accor-
dance with the strategies outlined above appear from Example 1.

20   In a further aspect, the present invention relates to an oligonucleo-
tide which is substantially identical to at least a subsequence of
one of the strands of the DNA sequence encoding EC-SOD C except for
the presence or absence of at least one nucleotide different from the
nucleotide at the same position of the DNA sequence encoding EC-SOD

25   C, which oligonucleotide is suitable for use in site-specific mutage-
nesis to be used in the preparation of a polypeptide of the invention
having the superoxide dismutating property of native EC-SOD and a
modified affinity for heparin compared to recombinant EC-SOD C. The
oligonucleotide is preferably of synthetic origin, i.e. prepared syn-

30   thetically by established standard methods as described above, and
has the characteristics described above. Examples of oligonucleotides
of the invention appear from Fig. 3.

Alternatively, the polypeptide of the invention having the superoxide
dismutating property of native EC-SOD and a modified affinity for

heparin compared to recombinant EC-SOD C may be prepared by a method comprising inserting a DNA sequence encoding native EC-SOD C into an expression vector, introducing said vector into a host cell capable of expressing EC-SOD C, growing said host cell under condition en-

5   suring the expression of substantial native EC-SOD C, subjecting the expressed substantial native EC-SOD C to a posttranslational modification so as to obtain the polypeptide of the invention, and recovering said polypeptide. The DNA sequence encoding native EC-SOD C is, e.g., the sequence of native EC-SOD C shown in Fig. 1, and the ex-

10   pression vector and host cell may be of any suitable type, e.g. of the types explained above.

Preferably, the posttranslational modification comprises chemical modification of the amino acid residues of the polypeptide sequence of native EC-SOD C, e.g. as explained above.

15   Any of the recombinant production methods outlined above may be carried out by use of higher organism as the host organism. Of particular interest is a production which is carried out by use of the well known transgenic techniques in which transgenic organisms such as animals are used as the host organism. Examples of suitable ani-

20   mals are sheep, cattle, pigs, etc. When transgenic techniques are used, the DNA encoding the polypeptide of the invention is suitably inserted into the genome of the animal in such a position that the polypeptide of the invention is expressed together with a polypeptide which inherently is expressed by the animal, preferably a polypeptide

25   which is easily recovered from the animal, e.g. a polypeptide which is secreted by the animal such as a milk protein or the like. Suitably, the DNA fragment encoding the polypeptide of the invention is inserted in the genome of the animal in frame with the DNA fragment encoding the polypeptide inherent to the animal so as to obtain

30   expression of a fusion protein comprising on the one hand the polypeptide of the invention and on the other hand the polypeptide related to the host organism, e.g. the animal. The resulting fusion protein may then be subjected to posttranslational modification, e.g. as described below, to obtain the polypeptide of the invention.

Alternatively, the polypeptide of the invention having the superoxide
dismutating property of native EC-SOD and a modified affinity for
heparin compared to recombinant EC-SOD C may be prepared by a method
comprising subjecting native EC-SOD C to an enzymatic or chemical

5   treatment, by which the native EC-SOD C is converted into a polypep-
tide of the invention having the formula set forth above or a modifi-
cation thereof having the superoxide dismutating property of native
EC-SOD.

Native EC-SOD C may be obtained from a cell line which is capable of

10   secreting EC-SOD C. The cell line may be of mammalian, in particular
human, origin. It is preferable that the cell line is one which pro-
duces particularly high quantities (compared to other cells) of EC-
SOD. Thus, the cell line may be one which is derived from blood or
lung, skin, blood vessel, pancreas, uterus, prostate gland, placenta

15   or umbilical cord tissue or, possibly, neoplastic tissue. In particu-
lar, it is contemplated that cell lines derived from fibroblasts or
glia cells are particularly advantageous as sources of EC-SOD.

Of course, and preferably, the native EC-SOD C to be used for the
preparation of the polypeptide of the invention may be of recombinant

20   origin produced by conventional recombinant techniques, e.g. as de-
scribed above. The recombinant production of native EC-SOD is descri-
bed in details in WO 87/01387.

The recombinantly produced polypeptide of the invention which is
expressed by cells may be secreted, i.e. exported through the cell

25   membrane, dependent on the type of cell and the composition of the
vector. If the polypeptide is produced intracellularly by the recom-
binant host, that is, is not secreted by the cell, it may be recover-
ed by standard procedures comprising cell disrupture by mechanical
means, e.g. sonication or homogenization, or by enzymatic or chemical

30   means followed by purification (examples of the purification and
recovery procedures are given in Examples 2, and 4-6).

In order to be secreted, the DNA sequence encoding the polypeptide
should be preceded by a sequence coding for a signal peptide, the
presence of which ensures secretion of the polypeptide from the cells

so that at least a significant proportion of the polypeptide expressed is secreted into the culture medium and recovered.

The polypeptide may be recovered from the medium by standard procedures comprising filtering off the cells and isolating the secreted
5   protein. In order to ensure the release of EC-SOD from the cell surfaces, and thus obtain an improved yield, it may be advantageous to add heparin or one of the substances with a similar effect mentioned above to the medium in which the cells expressing the polypeptide of the invention is grown.

10 As mentioned above, the polypeptide of the invention shows an affinity to heparin which indicates an affinity to heparan sulphate or other heparin-like glucosaminoglucans found on cell surfaces, especially on the surface of endothelial cells. It is therefore contemplated to induce the release of EC-SOD from cell surfaces and
15 thereby ensure an improved yield of EC-SOD by growing the tissue cells of the EC-SOD producing cell lines in a medium containing heparin or a heparin analogue, e.g. heparan sulphate, or another sulphated glucosaminoglycan, dextran sulphate or another strongly negatively charged compound in an amount which is sufficient to
20 induce the release of EC-SOD from the cell surfaces.

The polypeptide composition of the invention defined above may be prepared by a method comprising

a) cotransfecting expression vectors which separately comprise
25   the DNA sequence encoding each of the polypeptides to be included in the polypeptide composition by introducing each of the vectors into a host cell which is capable of expressing the polypeptides, culturing the host cell under conditions suitable for expressing each of the polypeptides to be included in the
30   polypeptide composition, and recovering and separating the culture medium comprising the polypeptide composition, or

b) coincubating a sample of each of the polypeptides to be included in the polypeptide composition under suitable conditions so as to obtain the polypeptide composition.

The cotransfection used for preparing the polypeptide composition of
the present invention may be carried out in accordance with tech-
niques known in the art, e.g. by the technique described in Example 2
below. The actual cotransfection method used is not critical as long

5   as it results in the introduction of the two expression vectors each
encoding a polypeptide of the invention.

Alternatively, one of the expression vectors may encode EC-SOD C to
be included in the polypeptide composition. The host organism or cell
into which the expression vectors are introduced may be any conven-

10   tional organism or cell which is capable of expressing the polypep-
tides and may e.g. be a mammalian cell line such as CHO. The cultiva-
tion of the host cell or organism and the recovering of the polypep-
tide composition may be carried out as described above.

The DNA sequences encoding the polypeptide constituents of the poly-

15   peptide composition of the invention may alternatively be carried by
the same expression vector in a manner which allows for the expres-
sion of both of the polypeptides either separately. The expression
vector carrying both DNA sequence may be introduced into the host
cell or organism by the normal procedure, e.g. as described above.

20   The coincubation of a sample of each of the polypeptides of the poly-
peptide composition serves to dissolve the oligomer form in question
of each of the polypeptides and recombine the resulting subunits and
may be performed by simply mixing polypeptide samples obtained as
described herein and subsequently incubating the mixture, e.g. with

25   gentle stirring, at a temperature which will not give rise to degra-
dation of the structure of the polypeptide, for example a temperature
in the range of 20-50°C or maybe even higher such as in certain cases
up to 60°C, often at a temperature of about 37°C, so as to achieve
recombination of the polypeptide species into mixed or heterologous

30   oligomers. Preferably, the polypeptides are dissolved in a physiolo-
gically acceptable solvent, such as water, or an aqueous physiologi-
cal acceptable solution such as α-medium, such as explained in the
examples.

The polypeptide of the invention having the superoxide dismutating
property of native EC-SOD and a modified affinity for heparin com-
pared to recombinant EC-SOD C may be recovered from the organism in
which it is produced or the medium to which it is secreted by adsorp-
5   tion to a matrix containing immobilized antibodies against the poly-
peptide of the invention, or against EC-SOD or an immunological de-
terminant thereof, eluting the EC-SOD activity from the matrix, and
pooling the fractions containing the EC-SOD activity, and optionally
subjecting the pooled fractions to further purification. The anti-
10   bodies employed may be antibodies raised against the polypeptide of
the invention, but may also be antibodies raised against EC-SOD or an
immunological determinant thereof, which antibodies, because of the
high degree of homology between the polypeptide of the invention and
EC-SOD, on most cases will react with the polypeptide of the inven-
15   tion (*vide* Example 8). The antibodies used for the recovery of the
polypeptide of the invention is immobilized on a suitable matrix
material in a manner known *per se*.

The antibodies employed for affinity chromatography according to the
invention may either be polyclonal or monoclonal antibodies. The
20   currently preferred antibodies are monoclonal antibodies as most
monoclonal antibodies have been found to bind the antigen less
strongly than the polyclonal antibody mixture which means that de-
sorption may be carried out under milder conditions with weaker
eluants. This may result in an improved yield of the polypeptide of
25   the invention as there is a lower degree of denaturation than when
strong eluants are used for desorption.

Also, since all the IgG will be directed against the polypeptide of
the invention, a far smaller amount of antibody matrix will have to
be used for the adsorption of the polypeptide from the material from
30   which it has to be recovered. The desorption of the polypeptide will
require a far smaller volume of eluant, thereby simplifying the
elution procedures which are at present inconvenient due to the large
volumes of eluant needed for the desorption.

The specificity of monoclonal antibodies for the polypeptide of the
35   invention is likely to be higher than that of the polyclonal anti-

bodies. The eluate from the antibody matrix will therefore be purer
which means that one or more further purification steps may be
omitted. This means that the production procedure will be greatly
simplified and a far higher yield will be obtained which presents an
5   important economic advantage.

Polyclonal antibodies may be obtained by immunizing an immunizable
animal with the polypeptide of the invention or an immunological
determinant thereof and obtaining antiserum such as immunoglobulins
from the animal in a manner known *per se*. The immunization is pre-
10 ferably performed by means of a stabilized aqueous solution of the
polypeptide; the stabilization agent may be a buffer such as phospha-
te buffered saline or an adjuvant (also to further increase the anti-
genicity), and a suitable adjuvant is Freund's adjuvant or aluminium
hydroxide. For immunization purposes, mice, rabbits, hens, goats and
15 sheep are the preferred animals. The bleeding of the animal and the
isolation of the antiserum is performed according to well-known
methods. The antibody is preferably provided in substantially pure
form which is advantageous in order to obtain the necessary purifica-
tion of the polypeptide of the invention.

20 When using a monoclonal antibody for the recovering of the polypep-
tide of the invention, it may be produced by a hybridoma technique
which is well-known method for producing antibodies. In the hybridoma
technique using, for instance, mice as the animals immunized, mice
are immunized with the antigen in question and spleen cells from the
25 immunized mice are fused with myeloma cells whereupon the fused
hybridoma cells are cloned, antibody-producing cells are grown in a
suitable growth medium and the antibodies are recovered from the
culture. The antibodies obtained by the hybridoma technique have the
advantage of greater specificity and, hence, a greater purifying
30 potential as mentioned above. In a possible further step, using
recombinant DNA techniques, the DNA sequence encoding the antibody is
transferred from the hybridoma cell clone to a suitable vector, the
hybrid vector is transformed to a suitable bacterial host, the host
is grown in an appropriate medium and the resulting antibody is
35 recovered from the culture. In this way, an improved yield of anti-
body may be obtained. The host may be one usually employed in the

field of recombinant DNA technology such as *Escherichia coli* or *Bacillus subtilis*.

In an alternative method of obtaining monoclonal antibodies, the hybridoma cells may be implanted and grown in the abdominal cavity of
5 mice, and the resulting antibodies against the polypeptide of the invention are recovered from the ascitic fluid produced, in a manner known *per se*. Furthermore, immunization for obtaining monoclonal antibodies may be performed *in vitro* using immunocompetent cells from, e.g., mice. In this case it is not necessary to inject an
10 antigen into the animal in question, e.g. a mouse, although this is at present the most commonly employed procedure. It should be noted that monoclonal antibodies and the method of preparing them form one aspect of the invention.

In most cases, especially when employing immobilized polyclonal anti-
15 bodies for the purification of the polypeptide of the invention, the pooled eluate of the antibody matrix may be absorbed to a matrix, e.g. an ion-exchange matrix, followed by eluting the EC-SOD activity from the matrix and pooling fractions containing the EC-SOD activity. Further purification of the pooled eluate may be obtained by applying
20 it on a chromatographic column of a matrix comprising heparin or a heparin analogue, e.g. heparan sulphate, or another sulphated glucosaminoglycan, dextran sulphate or another strongly negatively charged compound and eluting followed by pooling the fractions showing affinity to the substance in question.

25 The polypeptide of the invention which has been recovered by adsorption as described above is preferably eluted with an eluent such as NaCl, which has been found to be very efficient and which is also the eluent used for evaluating the heparin affinity of the polypeptide of the invention.

30 In a yet further, very important aspect, the present invention relates to the use of a polypeptide of the invention having the superoxide dismutating property of native EC-SOD and a modified affinity for heparin compared with recombinant EC-SOD C, for the diagnosis, prophylaxis or treatment of diseases or disorders connected with the

presence or formation of superoxide radicals and other toxic oxygen
intermediates derived from the superoxide radical.

Examples of such diseases or disorders are selected from conditions
involving ischaemia followed by reperfusion, e.g. infarctions such as

5   heart, kidney, brain, spinal cord, or intestinal infarctions; trans-
plantation of organs such as heart, lung, pancreas, liver, skin, bone
tissue, severed extremities, skeletal muscle; inflammatory diseases
such as rheumatoid arthritis, pancreatitis, in particular acute pan-
creatitis, pyelonephritis and other types of nephritis, and hepati-

10 tis, neuritis, uveitis, cystitis, peyronies disease, autoimmune dis-
eases, insulin-dependent diabetes mellitus, disseminated intravascu-
lar coagulation, fatty embolism, haemorrhagic shock, endotoxin-indu-
ced shock, septicemia, severe viral infections, adult respiratory
distress, infantile respiratory distress, brain haemorrhages in neo-

15 nates, burns, preservation of lens and cornea in the context of
transplantation, adverse effects of ionizing radiation, carcinogene-
sis, and adverse effects of toxins such as alloxan, paraquat, and
some cytostatic compounds.

Thus, the polypeptide of the invention is indicated for substantially

20 the same applications as CuZn SOD the therapeutic activity of which
has been more thoroughly documented as discussed below. EC-SOD and
the EC-SOD variants of the invention have, however, been found to
possess a number of characteristics which are assumed to make them
particularly useful for therapeutic applications. The property of the

25 new EC-SOD variants of the invention of having a heparin affinity
different from that of recombinant EC-SOD C is likely to confer upon
them a considerable advantage in terms of therapeutic usefulness, as
compared with recombinant EC-SOD C.

CuZn SOD has a low molecular weight (33,000) which causes it to be-

30 come eliminated very quickly by glomerular filtration in the kidneys
so that in rodents it has a plasma half-life of less than 10 minutes
and, in human beings, the enzyme has a half-life of about 20-30 mi-
nutes. EC-SOD C (K. Karlsson et al., *J. Clin. Invest. 82*, 1988, pp.
762-766) and the EC-SOD variants presented in this application (Exam-

35 ples 1, 2,& 4) have a much longer half-life. This is partly due to

the high molecular weight of EC-SOD and EC-SOD variants of the invention which prevents them from being eliminated by glomerular filtration, and partly due to the fact that EC-SOD and EC-SOD variants of the invention of the C-type seem to bind to endothelial cell sur-

5   faces (K. Karlsson et al., *Biochem. J. 242*, 1987, pp. 55-59; K. Karlsson et al., *Biochem. J. 255*, 1988, pp. 223-228; K. Karlsson et al., *J. Clin. Invest. 82*, 1988, pp. 762-766; K. Karlsson et al., *Lab. Invest. 60*, 1989, pp. 659-666). In the therapeutic use of EC-SOD and variants according to the invention, the enzyme therefore preferably

10   has a half-life in human beings of at least 4 hours and possibly even longer.

As explained above, EC-SOD is, in its native environment, a secreted protein and it is therefore likely that it is specifically synthesized for a function in extracellular space (in extracellular fluids

15   or on cell surfaces) which may cause it to exhibit properties which are particularly well adapted to protect plasma components or the outer surface of cells against the toxic effects of superoxide radicals or other oxygen radicals. A fundamental property of EC-SOD C, and most likely also a polypeptide of the invention having a slightly

20   reduced affinity for heparin compared to the heparin affinity of recombinant EC-SOD C, eg. the polypeptide of the invention T216 described herein (Examples 1, 2 and 4), is its affinity for heparan sulfate which *in vivo* apparently has the correlate of binding to heparan sulfate proteoglycan in the glycocalyx of cell surfaces and

25   in the interstitial connective tissue matrix (K. Karlsson et al., *Biochem. J. 242*, 1987, pp. 55- 59; K. Karlsson et al., *Biochem. J. 255*, 1988, pp. 223-228; K. Karlsson et al., *J. Clin. Invest. 82*, 1988, pp. 762-766; K. Karlsson et al., *Biochem. J. 256*, 1988, pp. 29-33; K. Karlsson et al., *Lab. Invest. 60*, 1989, pp. 659-666; K. Karlsson et

30   al., *Biochim. Biophys. Acta 967*, 1988, pp. 110-114). This property would seem to be a particularly efficient way to protect cells and tissues against an external superoxide radical source.

The significance of the apparent association of EC-SOD and EC-SOD variants of the invention with cell membranes is further supported by

35   the finding that CuZn SOD which has been modified with polylysine to bind to cell membranes is better able to protect activated (super-

oxide radical-producing) polymorphonuclear leukocytes (PMN) from
autoinactivation (cell death) than normal CuZn SOD which is nega-
tively charged and therefore tends to be repelled by the cell membra-
nes (M. Salin and J. M. Mc Cord, "Free radicals in leukocyte metabo-
5 lism and inflammation", in *Superoxide and Superoxide Dismutases, eds.*
A. M. Michelson, J. M. McCord and I. Fridovich, Academic Press, 1977,
pp. 257-270). The fact that Nocardia asteroides possesses a membrane-
associated SOD which appears to confer efficient protection against
activated human PMNs as the susceptibility of Nocardia to PMNs is
10 significantly increased when Nocardia cells are treated with anti-
bodies towards this SOD (B. L. Beaman et al., *Infect. Immun. 47,*
1985, pp. 135-141) also points to a cell membrane-protective function
of SOD bound to cell surfaces. Unlike EC-SOD and EC-SOD variants of
the invention, CuZn SOD has an intracellular function which may make
15 it less well suited for extracellular application, i.e. occasioned by
the extracellular presence of superoxide radicals. Furthermore, its
brief half-like compared to that of EC-SOD and EC-SOD variants of the
invention mentioned above would seem to make it necessary to admini-
ster larger doses at shorter intervals than is likely to be the case
20 with the EC-SOD variants of the invention.

SOD activity for potential therapeutic applications has been demon-
strated for the following diseases or disorders.

Parenterally administered CuZn SOD has been shown to exhibit an anti-
inflammatory effect in a series of animal models of inflammation as
25 well as in inflammatory diseases in animals (Huber and Saifer, in
Superoxide and Superoxide Dismutases, eds. Michelson et al., Academic
Press, 1977, pp. 517-549). In humans, positive effects of CuZn SOD
has been reported in rheumatoid arthritis and arthroses, in inflam-
mation of the bladder and other urological conditions (Menander-Huber
30 in *Biological and Clinical Aspects of Superoxide and Superoxide Dis-*
mutase, eds. Bannister et al., Elsevier/North Holland, 1980, pp. 408-
423) as well as adverse effects of treatment with ionizing radiation
(Edsmyr et al. *Current Therapy. Res. 10,* 1976, pp. 198-211; Cividalli
et al., *Acta. Radiol. Oncol. 24,* 1985, pp. 273-277 (in rats)). In
35 some countries, bovine CuZn SOD has become registered as a drug
(Orgotein, Peroxinorm), employed mainly for the treatment of arthri-

tis and arthroses where the composition is administered intraarticu-
larly (Goebel and Storck, *Am. J. Med. 74*, 1983, pp. 124-128), but
also for urological conditions.

Parenterally administered CuZn SOD is not taken up by the cells and
5   must exert its activity in the extracellular space. CuZn SOD encap-
sulated in liposomes is taken up by the cells and is reported to be
effective against Crohn's disease, Bechet's disease, dermatitis her-
petiformis, ulcerative colitis, Kawasaki's disease and against the
adverse effects of radiation therapy (Y. Niwa et al., *Free Rad. Res.*
10   *Comms. 1*, 1985, pp. 137-153). The mechanism of the anti-inflammatory
activity of CuZn SOD is not quite clear. Direct protection against
oxygen radicals formed by activated leukocytes has been suggested
(Halliwell, Cell Biol. *Int. Rep. 6*, 1982, pp. 529-541). Another
possibility is prevention of the formation of a superoxide induced
15   strongly chemotaxic substance (Petrone et al., *Proc. Natl. Acad. Sci.*
USA 77, 1980, pp. 1159-1163).

The other large potential area of application for SOD is as a protec-
tive factor against tissue damage caused by ischaemia followed by
reperfusion. If the supply of blood to a tissue is cut off, the tis-
20   sue will slowly become necrotic. Macro- and microscopically the da-
mage will typically develop slowly over many hours. If the tissue is
reperfused after, for instance, one hour, a strong acceleration of
the tissue damage will occur instead of an improvement. Most likely
there are several reasons for this so-called reperfusion paradox, but
25   oxygen radicals formed as a result of the reappearance of oxygen in
previously ischaemic tissue appear to contribute to the damage. The
radicals are extremely shortlived and therefore difficult to study
directly, and much of the information concerning their formation and
effects is inferred from the protective action of various oxygen
30   radical scavengers. Their formation have however also been demon-
strated more directly by means of EPR on heart samples (Zweier et
al., *J. Clin. Invest. 80*, 1987, pp. 1728-1734) and by means of spin
traps in heart reperfusion (e.g. P. B. Garlick et al., *Circ. Res. 61*,
1987, 751-760; R. Bolli et al., *J. Clin. Invest. 82*, 1988, 476-485).
35   Tissue protection by means of CuZn SOD has been demonstrated in
ischaemia- or anoxia-reperfusion models in the kidney (G. L. Baker et

al., *Am. Surg. 202*, 1985, pp. 628-641; I. Koyama et al., *Transplanta-tion 40*, 1985, pp. 590-595; E. Hansson et al., *Clin. Sci. 65*, 1983, pp. 605-610; A. Bayati et al., *Acta Physiol. Scand. 130*, 1987, pp. 367-372; J. F. Bennett et al., *Cryobiology 24*, 1987, pp. 264-269; T. Hoshino et al., *Transplantation 45*, 1988, pp. 284-289; P. J. Bosco et al., *Arch. Surg. 123*, 1988, pp. 601-604), intestine (D. A. Parks et al., *Gastroenterology 82*, 1982, pp. 9-15; M. H. Schoenberg et al., *Acta Chim. Scand. 150*, 1984, pp. 301-309; M. C. Dalsing et al., *J. Surg. Res. 34*, 1983, pp. 589-596; M. Younes et al., *Res. Exp. Med. 187*, 1987, pp. 9-17), pancreas (H. Sanfey et al., *Ann. Surg. 200*, 1983, pp. 405-413), liver (S. L. Atalla et al., *Transplantation 40*, 1985, pp. 584-589; G. Nordström et al., *Circ. Shock 26*, 1988, pp. 115-126), lung (R. S. Stuart et al., *Transplant. Proc. 17*, 1985, pp. 1454-1456; I. Koyama et al., I. Koyama et al., *J. Appl. Physiol.*, 63, 1987, pp. 111-115), skeletal muscle (R. V. Korthuis, *Circ. Res. 57*, 1985, pp. 599-609), skin (M. J. Im et al., *Ann. Surg. 201*, 1985, pp. 357-359; A. Sagi et al., *Plast. Reconstr. Surg. 77*, 1986, pp. 639-642; L. Huang et al., *FASEB J. 1*, 1987, pp. 129-132; T. J. Zimmerman et al., *Ann. Plast. Surg. 18*, 1987, pp. 218-223; A. T. Pokorny et al., *Arch. Otolar. Head Neck Surg. 115*, 1989, pp. 207-212), brain (J. P. Pigott et al., *J. Vasc. Surg. 7*, 1988, pp. 625-630; T. H. Liu et al., *Am. J. Physiol. 256*, 1989, pp. H589- H593), spinal cord (K. H. Lim et al., *Ann. Thorac. Surg. 42*, 1986, pp. 282-286; P. Cuevas et al., *Anat. Embryol. 179*, 1989, 251-255), and bone tissue (A.-P. C. Weiss et al., *Plast. Reconstr. Surg. 82*, 1988, pp. 486-495).

Preservation of heart function has been reported in isolated, per-fused preparations from the cat (M. Schlafer et al., *Circulation 66, Suppl. I*, 1982, pp. 185-192) and rat (Gaudel et al., *J. Mol. Cell Cardiol. 16*, 1984, pp. 459-470). In regional ischaemia-reperfusion models *in vivo*, reduction of infarct size has been reported in the dog (T. J. Gardner et al., *Surgery 94*, 1983, pp. 423-427; D. E. Chambers et al., *J. Mol. Cell Cardiol. 17*, 1985, pp. 145-152; S. W. Werns et al., *Circ. Res. 56*, 1985, pp. 895-898; G. Ambrosio et al., *Circulation 72*, 1986, pp. 1424-1433), in the pig (U. Näslund et al., *J. Mol. Cell Cardiol. 18*, 1986, pp. 1077-1084) and the rat (N. Aoki et al., *Br. J. Pharmacol. 95*, 1988, pp. 735-740). Protection by CuZn SOD has also been reported in models designed to mimic heart trans-

plantation (M. J. Jurmann et al., *J. Thorac. Cardiovasc. Surg. 95*, 1988, pp. 368-377; F. Gharagozloo et al., *J. Thorac. Cardiovasc. Surg. 95*, 1988, pp. 1008-1013; J. R. Stewart et al., *Ann. Thorac. Surg. 42*, 1986, pp. 390-393) and reperfusion after cardiopulmonary

5 bypass (F. Gharagozloo et al., *J. Thorac. Cardiovasc. Surg. 95*, 1988, pp. 631-636). Injection of SOD + catalase has also been reported to preserve the mechanical heart function after a brief (15 minutes) regional ischaemia in the dog (M. L. Myers et al., *Circulation 72*, 1985, pp. 915-921; K. Przyklenk and R. A. Kloner, *Circ. Res. 58*,

10 1986, pp. 148-156). Furthermore, SOD has been reported to reduce the incidence of ischaemia-and-reperfusion induced arrythmias (B. Woodward et al., *J. Mol. Cell. Cariol. 17*, 1985, pp. 485-493; M. Bernier et al., *Circ. Res. 58*, 1986, pp. 331-340; Tamwray et al., *Circ. Res. 63*, 1988, pp. 944-959). It is contemplated that EC-SOD and EC-SOD

15 variants of the invention might be used in connection with thrombolytic agents such as streptokinase, tissue plasminogen activator, urokinase and variants and variants of these factors. Enhanced myocardial salvage by the combined treatment with urokinase and CuZn SOD (U. Fincke et al., *Arzneim.-Forsch./Drug Res. 38*, 1988, pp. 138-142)

20 has been reported in a canine coronary thrombosis model.

Furthermore CuZn SOD has been shown to enhance the thrombolytic effects of tissue plasminogen activator in the dog heart. The result was hypothesized to be due to preservation of EDRF, resulting in reduced platelet aggregation and better vasodilation during reper-

25 fusion (J. L. Mehta et al., *Doctoral thesis, Faculty of Medicine, Upsaliensis, no. 215*, 1989, Acta Universitatis).

The source of oxygen radicals in this situation is not completely clear, but the effect of allopurinol seems to indicate that it is partly caused by xanthine oxidase which, by ischaemia, is converted

30 from its xanthine dehydrogenase form (Parks et al., op. cit.) to the radical-producing oxidase form. A large amount of hypoxanthine which is the substrate for xanthine oxidase is formed due to purine nucleotide degradation induced by ischaemia. Other sources of superoxide radicals may be activated leukocytes attracted to ischaemia-damaged

35 tissue, prostaglandin synthesis ($O_2 \cdot^-$ is a byproduct; Kontos, *Circ. Res. 57*, 1985, pp. 508-516) and autooxidation of various compounds

accumulated in reduced form during ischaemia. The finding concerning
ischaemia followed by reperfusion has potentially important clinical
applications. It may be possible to obtain an excellent effect by
reperfusion of tissue in connection with heart infarctions, by the
5   concomitant administration of a SOD such as EC-SOD or a polypeptide
of the invention and/or other protective factors against oxygen
radicals and thrombolytic factors, e.g. tissue plasminogen activator.
The results of the SOD experiments also indicate a possible applica-
tion in connection with heart surgery and heart transplantations.
10   Analogously, the results of employing an SOD in connection with
kidney ischaemia followed by reperfusion indicate that SOD may be
employed in connection with kidney transplantations and other organs
transplantations such as skin, lung, liver, pancreas, bone tissue
transplantations. Ischaemic brain disease is another possible indi-
15   cation.

*SODs show interesting protective effects in connection with other
pathological conditions.*

A variety of gastroenterological application are suggested by results
in the literature. Parenteral CuZn SOD ameliorates experimental ne-
20   crotizing enterocolitis in rabbits (D. A. Clark et al., *Am. J. Path.
130*, 1988, pp. 537- 542; M. J. S. Miller et al., *Am. J. Physiol. 255*,
1988, pp. G556-G565). A protection of gastric mucosa by parenteral
CuZn SOD against the effects of a variety of agents has been reported
in animal models; temporary reduction of celiac artery pressure (M.
25   A. Perry et al., *Gastroenterology 90*, 1986, pp. 362-367), haemorrha-
gic shock (C. von Ritter et al., *Dig. Dis. Sci. 33*, 1988, pp. 857864;
M. Itoh et al., *Gastroenterology 88*, 1985, 1162-1167), ethanol and
aspirin administration (G. Pihan et al., *Digest. Dis. Sci. 32*, 1987,
pp. 1395-1401), and E. coli sepsis (S. Arvidsson et al., *Circ. Shock
30   16*, 1985, pp. 383-393). The wound margin strength after intestinal
anastomosis is enhanced by parenteral CuZn SOD (H. Högström and U.
Haglund, *Surgery 99*, 1986, 716-720).

Hepatitis and hepatic injury experimentally induced by many means is
counteracted by parenteral CuZn SOD; ethanol (M. Younes et al., *Free
35   Rad. Res. Comms. 3*, 1987, pp. 19-26); corynebacterium parvum plus

endotoxin (M. J. P. Arthur et al., *Gastroenterology 89*, 1985, pp. 1114-1122); injection of galactoseamine and galactoseamine-latex particles (Y. Shiratori et al., *Hepatology 8*, 1988, pp. 815-821); injection of galactoseamine plus endotoxin (A. Wendel et al., *Bio-*

5   *chem. Pharmacol. 36*, 1987, pp. 2637-2639); and hypoxia (A. Yoshioka et al., *J. Dermatol. 14*, 1987, pp. 569-575).

Parenterally administered CuZn SOD also ameliorates acute pancrea-titis induced by infusion of oleic acid, partial obstruction of the excretory ducts and ischaemia followed by reperfusion (Sanfey et al.,

10   *Ann. Surg. 200*, 1984, pp. 405-413); and by cerulein-infusion (K. S. Gulce et al., *Am. J. Surg. 151*, 1986, pp. 163-169; A. Dabrowski et al., *Scand. J. Gastroenterol. 23*, 1988, pp. 1245-1249; J. Wisner et al., *Gut 29*, 1988, pp. 1516-1523). The results indicate the possi-bility of an active therapy against this disease for which no spe-

15   cific therapy exists at present.

It has also been suggested that treatment with SOD is effective against burns. The local oedema after an experimental slight burn in rats could be somewhat decreased through injection of SOD (Björk and Artursson, *Burns 9*, 1983, pp. 249-256). In an animal model involving

20   severe burn damage of mice a dramatic protection was obtained by means of SOD, where survival and local damage were concerned (Saez et al., *Circulatory Shock 12*, 1984, pp. 220-239). Burn-induced intra-vascular hemolysis is reduced by polyethyleneglycol- substituted CuZn SOD (Hatherill et al., *J. Clin. Invest.* 78, pp. 629-636) as is burn-

25   -induced myocardial depression (J. W. Horton et al., *J. Burn Care Rehabil. 9*, 1988, pp. 589-598).

In the case of, for instance, burns, immunocomplex formation, and major tissue damage, neutrophilic leukocytes are accumulated in the lungs. Complement activation (C5 a) often seems to mediate the accu-

30   mulation. The leukocytes seem to be activated and release oxygen radicals, thereby causing lung damage which, for instance, is charac-terized by increased vessel permeability and lung oedema (adult respiratory distress). In several animal models, SOD and other oxygen radical scavengers have been shown to have a protective effect

35   against lung damage (Till and Ward, *Agents and Actions, Suppl. 12*,

1983, pp. 383-396). Protection by CuZn SOD against other lung damage
models have also been reported; oedema formation during perfusion (B.
Risberg et al., *Eur. Surg. Res. 17*, 1985, pp. 230-236); intrabron-
chial installation of PMA (I. U. Schraufstätter et al., *J. Clin.*
5   *Invest. 73*, 1984, pp. 1175-1184; J. S. Kerr et al., *Agent. Action.*
21, 1987, pp. 293-296); xanthine oxidase (O. D. Saugstad et al.,
Intensive Care Med. 13, 1987, pp. 30-32); or hyperoxia R. V. Pad-
manabhan et al., *Am. Rev. Respir. Dis. 132*, 1985, pp. 164-167).

Parenterally administered CuZn SOD has been reported to prevent
10 bronchopulmonary dysplasia in preterm neonates suffering from infan-
tile respiratory distress (W. Rosenfeld et al., *J. Pediatr. 105*,
1984, pp. 781-785).

Concerning the central nervous system protective effects of CuZn SOD
have been shown against posttraumatic brain oedema (P. H. Chan et
15 al., *Ann. Neurol. 21*, 1987, pp. 540-547) and experimental autoimmune
neuritis (H.-P. Hartung et al., *Ann. Neurol. 23*, 1988, pp. 453-460).
In a beagle puppy model, injection of SOD has been reported to reduce
the frequency of intraventricular brain haemorrhage following hypo-
tension (L. R. Ment et al., *J. Neurosurg. 62*, 1985, J63-J69).

20 The endothelium-derived vessel relaxant factor (EDRF) is very sensi-
tive to superoxide, and administration of SOD augments its actions
(R. J. Gryglewski et al., *Nature 320*, 1986, pp. 454-456; G. M. Ruba-
nyi et al., *Am. J. Physiol. 250*, 1986, pp. H822-H827). Superoxide
radical production can occur under many circumstances in the body and
25 may cause vasoconstriction and decreased tissue perfusion. Admini-
stration of SOD is believed to be able to relieve such vasoconstric-
tion and also to enhance other effects of EDRF such as platelet
stabilization.

Acute severe increase in blood pressure leads to functional and mor-
30 phologic abnormalities in brain arterioles. Prostaglandin synthesis
inhibitors and superoxide dismutase is contemplated to protect
against the abnormalities. Superoxide release can be detected (H. A.
Kontos, *Circ. Res. 57*, 1985, pp. 508-516). Close analysis of the
model has lead to the conclusion that superoxide radicals are formed

as a byproduct during prostaglandin synthesis. The results suggest
that tissue damage caused by superoxide radicals released during pro-
staglandin synthesis may occur in other pathological situation and
that SOD may exert a protective action.

5   CuZn SOD + catalase in the medium have been reported to prolong the
survival of the perfused isolated rabbit cornea (O. N. Lux et al.,
Curr. Eye Res. 4, 1985, pp. 153-154). CuZn SOD + catalase protect the
isolated lens against photoperoxidation (S. D. Varma et al., *Ophthal-
mic Res. 14*, 1982, pp. 167-175). The results suggest possible bene-
10 ficial effects of SOD in cornea transplantations and other ophthalmic
surgical procedures. Parenteral CuZn SOD protects against uveitis
induced by injection of lens protein (N. A. Rao et al., *Ophthalmic
Res. 18*, 1986, pp. 41-46) and soluble retinal antigen (N. A. Rao et
al., *Invest. Ophthalmol. Visual Sci. 28*, 1987, pp. 886-892).

15 Ameliorative action of parenteral SOD has been reported in animal
models of such acute conditions as disseminated intravascular coa-
gulation (T. Yoshikawa et al., *Thromb. Haemostas. 50*, 1983, pp. 869-
872), septicemia (H. F. Welter et al., *Chirurgisches Forum '85 f.
experim. u. klinischer Forschung, Springer- Verlag, Berlin*, 1985),
20 and endotoxin-induced shock (C. W. Broner et al., *Crit. Care Med.
16*, 1988, pp. 848-850).

Parenteral SOD has been reported to enhance survival in an acute
viral infection in mice (T. Oda et al., *Science 244*, 1989, 974-976).

In various types of autoimmune disease, such as systemic lupus
25 erythematosus (SLE), systemic sclerosis and rheumatoid arthritis, an
increased frequency of chromosomal breaks in lymphocytes has been
demonstrated (Emerit, "Properties and action mechanisms of clasto-
genic factors", in *Lymphokines, Vol. 8, ed. E. Pick, Academic Press*,
1983, pp. 413-424). Fibroblast cultures and direct bone marrow prepa-
30 rations also sometimes show increased breakage. Plasma from such
patients contains a chromosome breaking - clastogenic - factor. In
some instances a similar factor has also been demonstrated in syno-
vial fluid and in cerebrospinal fluid (disseminated sclerosis).
Breaks occur in normal lymphocytes which are cocultivated with lym-

phocytes from patients with autoimmune disease. Lymphocytes from
patients condition culture media to produce chromosome breaks. The
clastogenic activity of SLE plasma can be increased by UV-irradia-
tion. Production of superoxide in plasma by means of xanthine oxidase

5   and xanthine results in formation of clastogenic activity. In all the
above described models, addition of CuZn SOD to the medium protected
against the clastogenic activity (Emerit, ibid.). This indicates that
superoxide radicals are involved in both the formation and actions of
the clastogenic factor (Emerit, ibid.). In an animal model of SLE,

10   the New Zealand black mouse which possesses the clastogenic factor,
the chromosomes are protected in bone marrow cells *in vivo* by repea-
ted injections of SOD (Emerit et al., *Cytogenet. Cell Genet. 30*,
1982, pp. 65-69). It is, however, still unclear to what extent the
clastogenic factor contributes to the major symptoms in human auto-

15   immune disease and whether administration of SOD has any therapeutic
effect.

The neoplastic transformation of cells is usually divided into two
phases, i.e. initiation followed by promotion. In *in vitro* models
where initiation has been caused by ionizing radiation, bleomycin,

20   misonidazole and other nitroimidazoles, the oncogenic transformation
has been effectively inhibited by the presence of SOD in the medium.
It is not necessary for SOD to be present during exposure to the
initiating substances which seems to indicate that the enzyme inhi-
bits the subsequent promotion step (Miller et al., *Int. J. Radiat.*

25   *Oncol. Biol. Phys. 8*, 1982, pp. 771-775; Borek and Troll, *Proc. Nat.*
Acad. Sci. USA 80, 1983, pp. 1304-1307). Non-toxic doses of xanthine
+ xanthine oxidase causes promotion in growing cells. Addition of SOD
or SOD + catalase inhibits this effect (Zimmerman and Cerutti, *Proc.*
Nat. Acad. Sci. USA 81, 1984, pp. 2085-2087). Phorbol esters are

30   known promoters. In a model in which skin tumors were induced by
initiation with a benzanthracene followed by application of a phorbol
ester (TPA), local treatment with a lipophilic copper complex with
SOD activity strongly reduced tumor formation (Kenzler et al., *Scien-*
ce 221, 1983, pp. 75-77). The result indicates that, at least in cer-

35   tain cases, superoxide radicals contribute to the promotion of tumor
formation and that SOD may protect against this effect.

There is reason to believe that oxygen radicals contribute to the
damaging effects of a number of toxic substances such as bleomycin,
adriamycin, alloxan, 6-hydroxydopamine, paraquat, dihydroxyfumaric
acid, nitrofurantoin and streptozotocin. In those cases where radical

5   formation takes place in the extracellular space it might be possible
to protect by means of injected protective enzyme. Thus, SOD may
protect against the diabetogenic activity of alloxan (damages β-cells
in the pancreas) *in vitro* (Grankvist et al., *Biochem. J. 182*, 1979,
pp. 17-25) and *in vivo* (Grankvist et al., *Nature 294*, 1981, pp. 158-

10   160). The damaging effect of alloxan seems therefore to be mediated
by the superoxide radical or by other oxygen radicals derived from
it. The reason for the great sensitivity of the β-cells to alloxan is
not clear, and it has been speculated whether there is any connection
between alloxan sensitivity and the incidence of insulin-dependent

15   diabetes mellitus. In diabetes mellitus there is an infiltration in
the Langerhans' islets by inflammatory cells which potentially may
form oxygen radicals. It may therefore be contemplated to protect the
β-cells by injections with SODs such as EC-SOD or a polypeptide of
the invention at the first onset of diabetes mellitus.

20   It has been reported (Mossman and Landesman, *Chest 835*, 1983, pp.
50s-51s) that SOD added to the growth medium protects tracheal cells
against asbestos.

It has been described (Roberts et al., *J. Urol. 128*, 1982, pp. 1394-
1400; A. Kaur et al., *Biochem. Int. 16*, 1988, pp. 1083-1094) that

25   parenteral CuZn SOD protects kidneys against experimentally induced
pyelonephritis. CuZn SOD protects against acute nephritis induced in
rats by anti-glomerular basement membrane antibodies (A. Rehan et
al., *Lab. Invest. 51*, 1984, pp. 396-403), and nephrotoxic serum (T.
Adachi et al., *Biochem. Pharmacol. 35*, 1986, pp. 341-345). Further-

30   more a protection against aminonucleoside nephritis has been demon-
strated (J. R. Diamond et al., *Kidney Int. 29*, 1986, pp. 478-483; M.
Beaman et al., *Clin. Sci. 73*, 1987, pp. 329-332).

Generally, CuZn SOD has been employed as the test substance in the
experiments described above. It is, however, assumed that EC-SOD

35   variants of the invention may be employed for the same purposes and,

as has been indicated above, with greater efficiency due to its
particular properties which may make it especially attractive to
employ EC-SOD variants of the invention extracellularly.

Recombinant EC-SOD C has been found to be more efficient than CuZn
5   SOD in disease models in which the two SODs have been tested in
parallel. Thus, as described by Johansson et al., *Cardiovascular
Research 24*, 1990, pp. 500-503, recombinant EC-SOD C has been shown
to reduce the concentration of oxygen-free radicals in reperfused rat
hearts. The effect of rEC-SOD C in reducing the free radical concen-
10   trations was concluded to be at least of the same extent as CuZn SOD.
Furthermore, recombinant human EC-SOD C has been shown to reduce myo-
cardial damage in rats subjected to ischemia and 24 hours of reperfu-
sion (G. Wahlund et al., *J. Mol. Cell. Cardiol. 22*, Suppl. 1 III,
1990, p. 47). In contrast, CuZn SOD did not show a significant induc-
15   tion of myocardial damage 24 hours after onset of reperfusion. M.
Erlansson et al., *Free Rad. Biol. Med.*, in press 1990 discloses the
use of EC-SOD C and bovine CuZn SOD as an inhibitor of post-ischemic
microvascular permeability increase in hamsters.

The present invention further relates to a pharmaceutical composition
20   which comprises a polypeptide of the invention having the superoxide
dismutating property of native EC-SOD and a modified affinity for
heparin compared with recombinant EC-SOD C together with a pharmaceu-
tically acceptable excipient, diluent or vehicle. The polypeptide of
the invention incorporated in the composition may be from any of the
25   sources discussed above, i.e. of recombinant, cell line or tissue
origin.

The estimate of a suitable, i.e. therapeutically active, dosage for
systemic treatment is made on the basis of the content of EC-SOD in
the human body. EC-SOD is the major SOD in human plasma, and the
30   total activity (composed of fractions A, B, and C; K. Karlsson et
al., *Biochem. J. 242*, 1987, pp. 55-59) is about 20 U/ml. Injection of
200 IU heparin per kg body weight results in an increase of EC-SOD
fraction C of about 23 U/ml (K. Karlsson et al., *Biochem. J. 242*,
1987, pp. 55-59). Although this heparin dosage is very high, a maxi-
35   mum release was apparently not achieved. In the pig in which a maxi-

mal EC-SOD C release could be obtained with a very high heparin dose, 200 IU heparin per kg body weight resulted in a 50 % of that maximal EC-SOD C release (K. Karlsson et al., *Biochim. Biophys. Acta 967*, 1988, pp. 110-114). Assumption of a similar dose-response relation-

5   ship in man results in an estimate of total EC-SOD of about 66 U/ml plasma (20+2x23 U/ml). The total plasma volume is about 4.7 % of the body weight corresponding to about 3.3 1 in a 70 kg person. 1 unit EC-SOD equals about 8.8 ng. The total amount of EC-SOD in the blood vessels (plasma and vessel endothelium) is therefore 3,300 x 66 x 8.8

10   x 10^{-9} g = 1.92 mg. A tenfold increase would require 19 mg and a 300-fold increase 575 mg EC-SOD C. A suitable dosage of polypeptide of the invention may therefore be in the range of about 15-600 mg/day, dependent, i.e. on the type and severity of the condition for which administration of EC-SOD is indicated. Injection of, for in-

15   stance, 87 mg EC-SOD C (a 50-fold increase) would result in 26 μg/ml in plasma (disregarding endothelium binding). This or even lower con- centrations show strong protective properties in *in vitro* experiments (with CuZn SOD) (cf. A. Baret, I. Emerit, *Mutation Res. 121*, 1983, pp. 293-297; K. Grankvist, S. Marklund, J. O. Sehlin, I. B. Täljedal,

20   *Biochem. J. 182*, 1979, pp. 17-25).

The dosage and timing of polypeptide of the invention injections depends on the half-life of the enzyme in human blood vessels, which is not yet known. It may as in rabbits be about 15 h (K. Karlsson et al., *J. Clin. Invest. 82*, 1988, pp. 762-766). The half-life in humans

25   is however probably longer. Assuming first-order kinetics and a half- life of 36 h, daily injections of 35 mg after an initial injection of 87 mg would therefore result in the same concentration as after the initial injection.

EC-SOD C (K. Karlsson et al., *Biochem. J. 242*, 1987, pp. 55-59) and a

30   polypeptide of the invention having a slightly reduced affinity for heparin compared to the heparin affinity of recombinant EC-SOD C, e.g. the polypeptide T216 of the invention described herein (Examples 1, 2 and 4) can be mobilized from cell surfaces to plasma with hepa- rin. Parenteral heparin, other sulphated glucosaminoglycans and other

35   strongly negatively charged substances may be used to modulate the location of endogenous or injected EC-SOD C and a polypeptide of the

invention, having slightly modified affinity for heparin compared to
recombinant EC-SOD C. Localization to the plasma phase might be use-
ful in certain pathological conditions.

Except for type C (K. Karlsson et al., *Biochem. J. 242*, 1987, pp.
5   55-59; K. Karlsson et al., *Biochem. J. 255*, 1988, pp. 223-228) the
structure of EC-SOD of type A and of type B is unknown. As shown in
the present application and as stated above, variations in heparin
affinity can be induced by mutations in the carboxyterminal end of
recombinant EC-SOD C. A minor truncation resulted in a polypeptide
10   having a slightly reduced heparin affinity i.e. EC-SOD variant T216
of the invention and further minor truncation resulted surprisingly
in loss of heparin affinity (A types). B-types could instead be con-
structed by means of making heteropolymers between a polypeptide
having an affinity for heparin corresponding to the C-type affinity,
15   a polypeptide having a slightly reduced affinity as compared with C-
type affinity and a polypeptide which does not display heparin bind-
ing. Since the results now establish that the binding to heparin is
dependent on the C-terminal end, it is contemplated that further
variation (increase or decrease) of the heparin affinity can be
20   brought about by exchanges (mutations) of amino acids or other chan-
ges in this region of EC-SOD.

The therapeutic usefulness of the polypeptides of the invention may
vary according to the particular disease to be treated. When strong
binding to cell-surfaces is advantageous EC-SOD C or the polypeptides
25   of the invention with even stronger heparin affinity may be most
useful, for example in transplantation of organs or if a polypeptide
of the invention is to be injected into an inflamed organ.

In yet other conditions a more limited heparin affinity such as that
of a polypeptide having a slightly reduced affinity e.g the polypep-
30   tide of the invention, T216, described herein, may be advantageous.
In a situation in which the time of reperfusion cannot be predicted
precisely (e.g. thrombolysis of coronary artery or artery in other
organs) a strong binder such as EC-SOD C injected before reperfusion
would bind firmly to endothelium around the body and very little
35   would be available to protect immediately the reperfused organ ter-

ritory at reperfusion. A polypeptide having a slightly reduced affi-
nity e.g the polypeptide of the invention, T216, described herein,
would in this situation exist in relatively high concentration in the
plasma phase (Examples 4.1 and Fig. 13), but would still bind endo-

5   thelium and is also likely to redistribute more rapidly between bind-
ing sites in organs. The work of (K. Karlsson et al., *Biochem. J.
255*, 1988, pp. 223-228), especially Fig. 3, suggests that only EC-SOD
variants with a heparin affinity of a magnitude which results in
elution from Heparin-Sepharose® column at a NaCl concentration of

10   0.55 M or higher (under the conditions stated in the Analytical
Methods section below) will bind to endothelium *in vivo*. Such vari-
ants may be advantageous in conditions where some but variable degree
of cell-surface binding is valuable.

Variants which apparently do not bind heparan sulfate *in vivo*, A and

15   B-types, may finally be useful when a high SOD activity in plasma or
the interstitial fluid phase is important.

For topical treatment, far less of a polypeptide composition of the
invention as described above would probably be needed. At present,
4-8 mg of CuZn SOD are administered intraarticularly once a week for

20   the treatment of arthritis. EC-SOD which has a far higher molecular
weight is likely to remain in the joint for a longer period of time.
A similar treatment protocol or possibly somewhat lower doses will
probably be appropriate.

DESCRIPTION OF THE DRAWINGS

The invention is further described with reference to the drawings, in
which

Fig. 1A, Fig. 1B and Fig. 1C show the cDNA sequence and deduced amino

5   acid sequence of EC-SOD C which has been obtained as in WO 87/01387.
       In Fig. 1A, the introduced translational stop codons of the EC-SOD
       variants T209, T213, T215 and T216 are identified by asterisks. In
       Fig. 1B, the amino acids introduced by substitutions in the EC-SOD
       variants G1, SA216, SA219 and SA220 are identified. In Fig. 1C, the

10   amino acid introduced by substitution and the introduced translatio-
       nal stop codon in the EC-SOD variant SAT216 are identified.

       Fig. 2 shows the structure of plasmid pPS3 used for producing each of
       the EC-SOD variants T216, T215, T213, T209, SA216, SA219, SA220 and
       G1. The white areas represent SV40 DNA and the numbers refer to the

15   corresponding nucleotide positions in SV40. SV40PE and SV40 origin
       represent the SV40 early promotor and the SV40 origin of replication,
       respectively, and arrows show the direction of transcription and DNA
       replication, respectively. SV40 polyadenylation signals are located
       between positions 2770 and 2533. The hatched area represents the DNA

20   sequence encoding each of the EC-COD variants T216, T215, T213, T209
       and G1, respectively. Dotted area represents polylinker DNA. The
       solid black area represents the β-lactamase gene (AP^R) of plasmid
       pBR322. Thin lines represent plasmid pBR322 DNA. Also indicated is
       the location of the pBR322 origin of replication.

25   Fig. 3 shows the sequences of the oligonucleotides used to produce
       the EC-SOD variants T216, T215, T213, T209, SA216, SA219, SA220 and
       G1 by site-directed mutagenesis as explained in Example 1. Also shown
       are the sequences and the nucleotide positions of the corresponding
       parts of the EC-SOD C template. Stars indicate the introduced stop

30   codons in oligonucleotides T216, T215, T213 and T209 and, in the case
       of SA216, SA219, SA220 and G1, the introduced codon for alanine and
       glutamine, respectively.

Fig. 4 is a graph showing the analytical separation of variant T216
by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant T216 was separated on Heparin-Sepharose®, as de-
scribed below in the Analytical Methods section, and EC-SOD content

5   (right abscissa) of the fractions (□) determined by ELISA, as de-
scribed below in the Analytical Methods section. The unbroken line
represents absorbance at 280 nm (left abscissa). The dotted line
represents the NaCl gradient (right abscissa).

Fig. 5 is a graph showing the analytical separation of variant T215

10   by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant T215 was separated on Heparin-Sepharose®, as de-
scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA, as de-
scribed below in the Analytical Methods section. The unbroken line

15   represents absorbance at 280 nm (left abscissa). The dotted line
represents the NaCl gradient (right abscissa).

Fig. 6 is a graph showing the analytical separation of variant T213
by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant T213 was separated on Heparin-Sepharose®, as de-

20   scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA, as de-
scribed below in the Analytical Methods section. The unbroken line
represents absorbance at 280 nm (left abscissa). The dotted line
represents the NaCl gradient (right abscissa).

25   Fig. 7 is a graph showing the analytical separation of variant T209
by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant T209 was separated on Heparin-Sepharose®, as de-
scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA, as de-

30   scribed below in the Analytical Methods section. The unbroken line
represents absorbance at 280 nm (left abscissa). The dotted line
represents the NaCl gradient (right abscissa).

Fig. 8A is a graph showing the analytical separation of variant SA216
by Heparin-Sepharose® chromatography. Culture medium of Example 2

containing variant SA216 was separated on Heparin-Sepharose® as de-
scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA as descri-
bed below in the Analytical Methods section. The unbroken line repre-
5   sents absorbance at 280 nm (left abscissa). The dotted line repre-
sents the NaCl gradient (right abscissa).

Fig. 8B is a graph showing the analytical separation of variant SA219
by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant SA219 was separated on Heparin-Sepharose® as de-
10   scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA as descri-
bed below in the Analytical Methods section. The unbroken line repre-
sents absorbance at 280 nm (left abscissa). The dotted line repre-
sents the NaCl gradient (right abscissa).

15   Fig. 8C is a graph showing the analytical separation of variant SA220
by Heparin-Sepharose® chromatography. Culture medium of Example 2
containing variant SA220 was separated on Heparin-Sepharose® as de-
scribed below in the Analytical Methods section, and EC-SOD content
(right abscissa) of the fractions (□) determined by ELISA as descri-
20   bed below in the Analytical Methods section. The unbroken line repre-
sents absorbance at 280 nm (left abscissa). The dotted line repre-
sents the NaCl gradient (right abscissa).

Fig. 8D is a graph showing the analytical separation of variant G1 by
Heparin-Sepharose® chromatography. Culture medium of Example 2 con-
25   taining variant G1 was separated on Heparin-Sepharose®, as described
below in the Analytical Methods section, and EC-SOD content (right
abscissa) of the fractions (□) determined by ELISA, as described
below in the Analytical Methods section. The unbroken line represents
absorbance at 280 nm (left abscissa). The dotted line represents the
30   NaCl gradient (right abscissa).

Fig. 8E is a graph showing the analytical separation of variant
SAT216 by Heparin-Sepharose® chromatography. Culture medium of Exam-
ple 2 containing variant SAT216 was separated on Heparin-Sepharose®
as described below in the Analytical Methods section, and EC-SOD

content (right abscissa) of the fractions (□) determined by ELISA as
described below in the Analytical Methods section. The unbroken line
represents absorbance at 280 nm (left abscissa). The dotted line
represents the NaCl gradient (right abscissa).

5   Fig. 9 is a graph showing the analytical separation by Heparin-
     Sepharose® chromatography of EC-SOD variants obtained when plasmids
     pPS3 and pPST213 were cotransfected into CHO DXB11 cells. Culture
     medium of Example 2 containing the variants was separated on Heparin-
     Sepharose®, as described below in the Analytical Methods section, and
10   EC-SOD content (right abscissa) of the fractions (□) determined by
     ELISA, as described below in the Analytical Methods section. The un-
     broken line represents absorbance at 280 nm (left abscissa). The
     dotted line represents the NaCl gradient (right abscissa).

     Fig. 10 is a graph showing the analytical separation by Heparin-
15   Sepharose® chromatography of EC-SOD variants obtained when plasmids
     pPST216 and pPST213 were cotransfected into CHO DXB11 cells. Culture
     medium of Example 2 containing the variants was separated on Heparin-
     Sepharose®, as described below in the Analytical Methods section and
     EC-SOD content (right abscissa) of the fractions (□) determined by
20   ELISA, as described below in the Analytical Methods section. The un-
     broken line represents absorbance at 280 nm (left abscissa). The
     dotted line represents the NaCl gradient (right abscissa).

     Fig. 11 is a graph showing the analytical separation by Heparin-
     Sepharose® chromatography of EC-SOD variants obtained when recom-
25   binant EC-SOD C and variant T213 were coincubated for various times
     as described in Example 5.3. The culture medium containing the vari-
     ants was separated on Heparin-Sepharose®, as described below in the
     Analytical Methods section, and EC-SOD content (right abscissa) of
     the fractions determined by ELISA, as described below in the Analyti-
30   cal Methods section. The figure shows as examples the pattern obtai-
     ned just after mixing (*) and after 23 hrs of incubation (□). The
     unbroken line represents absorbance at 280 nm (left abscissa) and the
     dotted line represents the NaCl gradient (right abscissa).

Fig. 12 is a graph showing plasma content of recombinant EC-SOD C
following its intravenous bolus injection in rabbits. About 500 μg/kg
body weight pure recombinant EC-SOD C (K. Hjalmarsson et al., *Proc.
Natl. Acad. Sci. USA. 84*, 1987, pp. 6340-6344) was injected into

5   three rabbits as described in Example 3 and blood samples drawn at
the times indicated for analysis of EC-SOD content. In addition, at
20 minutes, 300 minutes and 24 hours following recombinant EC-SOD C
administration, heparin 2,500 IE/kg body weight was injected intrave-
nously (indicated by arrows) and plasma EC-SOD determined at short

10   intervals thereafter. Each symbol represents one individual rabbit.

Fig. 13 is a graph showing plasma content of variant T216 following
its intravenous bolus injection in rabbits. About 15 to 20 μg/kg body
weight of variant T216 was injected into 5 rabbits as described in
Example 4.1. The variant T216 employed was partially purified by

15   means of Q-Sepharose®, Phenyl-Sepharose® and Heparin-Sepharose® as
described in Example 6. Blood samples were drawn at the times indi-
cated for analysis of EC-SOD content. In addition, at 25 minutes,
120 minutes, 300 minutes and 24 hours following administration of
variant T216, heparin 2,500 IE/kg body weight was injected intrave-

20   nously (indicated by arrows) and plasma EC-SOD determined at short
intervals thereafter. Each symbol represents one individual rabbit.

Fig. 14 is a graph showing plasma content of variant T213 following
its intravenous bolus injection in rabbits. About 20 μg/kg body
weight of variant T213 was injected into 2 rabbits as described in

25   Example 4.3. The variant T213 employed was partially purified by
means of CNBr-activated-Sepharose®, Q-Sepharose®, and Heparin-Sepha-
rose®, as described in Example 8. Blood samples were drawn at the
times indicated for analysis of EC-SOD content. In addition, at 300
minutes and 24 hours following administration of variant T213, he-

30   parin 2,500 IE/kg body weight was injected intravenously (indicated
by arrows) and plasma EC-SOD determined at short intervals there-
after. Each symbol represents one individual rabbit.

Fig. 15 is a graph showing the separation of variant T216 on Q-Sepha-
rose®. Culture medium containing variant T216 was applied to a

Q-Sepharose® column, as described in Example 6.1. EC-SOD content
(right abscissa) of the fractions (□) was determined by ELISA, as de-
scribed below in the Analytical Methods section. The unbroken line
represents absorbance at 280 nm (left abscissa). The activity was
5   pooled as indicated in the figure.

Fig. 16 is a graph showing the separation of variant T216 on Phenyl-
Sepharose®. The pool from the Q-Sepharose® separation step (Fig. 14)
was applied to a Phenyl-Sepharose® column, as described in Example
6.2. EC-SOD content (right abscissa) of the fractions (□) was deter-
10   mined by ELISA, as described below in the Analytical Methods section.
The unbroken line represents absorbance at 280 nm (left abscissa).
The activity was pooled as indicated in the figure.

Fig. 17 is a graph showing the separation of variant T216 on Heparin-
Sepharose®. The pool from the Phenyl-Sepharose® separation step (Fig.
15   15) was applied to a Heparin-Sepharose® column, as described in Exam-
ple 6.3. EC-SOD content (right abscissa) of the fractions (□) was
determined by ELISA, as described below in the Analytical Methods
section. The unbroken line represents absorbance at 280 nm (left
abscissa). The dotted line represents the NaCl gradient (right ab-
20   scissa). The activity was pooled as indicated in the figure.

Fig. 18 is a photograph showing the electrophoresis of recombinant
EC-SOD, variant T216 and variant T213 in a polyacrylamide gel, as de-
scribed below in Examples 7 & 8. The samples were denatured (10 mi-
nutes, 100°C) in solution containing sodium dodecylsulfate and 2-
25   mercaptoethanol. About 1 μg of each enzyme was applied to a gradient
(10 to 17.5%) polyacrylamide gel equilibrated with sodium dodecyl-
sulfate. The gel was stained with Coomassie Brilliant Blue. A crude
Bovine Serum Albumin preparation was added to recombinant EC-SOD C
and variant T213.

Lane A   Molecular weight standards (Phosphorylase b, 94 kDa;
          Bovine Serum Albumin, 67 kDa; Ovalbumin, 43 kDa: Carbonic
          Anhydrase, 30 kDa; Soybean Trypsin Inhibitor, 20 kDa
Lane B   Recombinant EC-SOD C
5   Lane C   Variant T216
Lane D   Variant T213

Fig. 19 is a photograph showing Western blot of recombinant EC-SOD
C, variant T216 and variant T213. The samples were first electropho-
resed on a gradient (10 to 17.5%) polyacrylamide gel as described in
10   the legend to Fig. 18, except that only 25-50 ng of each enzyme was
applied. The gel was then blotted onto a Immobilon PVDF transfer
membrane using a Bio-Rad Electroblotter. The membrane was saturated
for 1 hour at 37°C with a blocking buffer containing 5% non fatty
milk. The membrane was then incubated for 2 hours with a polyclonal
15   rabbit anti-human EC-SOD antibody (6.6 μg/ml) in buffer containing
2.5% non fatty milk. Then, the membrane was incubated with an alka-
line phosphatase-conjugated porcine anti-rabbit antibody (Dakopatts
D-306) in buffer containing 25% non fatty milk. The gel was finally
developed in solution containing 0.34 mg/ml nitroblue tetrazolium,
20   0.315% dimethylformamide, and 0.175 ng/ml 5-bromo-4-chloro-3-indolyl
phosphate, toluidinium salt).

Lane A   25 ng of recombinant EC-SOD C;
Lane B   25 ng of variant T216;
Lane C   25 ng of variant T213;
25   Lane D   50 ng of recombinant EC-SOD C;
Lane E   50 ng of variant T216; and
Lane F   50 ng of variant T213.

Fig. 20 is a graph showing the separation of variant T213 on Q-Sepha-
rose®. The variant T213 preparation, previously passed through an
30   Anti EC-SOD-Sepharose® column, was applied to a Q-Sepharose® column,
as described in Example 8.1. EC-SOD content (right abscissa) of the
fractions (□) was determined by ELISA, as described below in the
Analytical Methods section. The unbroken line represents absorbance
at 280 nm (left abscissa). The activity was pooled as indicated in
35   the figure.

MATERIAL AND METHODS USED IN THE EXAMPLES

Material

All chemicals (NaCl, H_2O_2, Na_2CO_3, H_2SO_4, potassium phosphate, sodium
5 citrate, sodium phosphate, sodium cacodylate buffer, sodium dodecyl-
sulfate, O-phenylenediamine, 1-aminoethylpropanol hydrochloride and
EDTA) were of analytical grade.

Restriction enzymes were supplied from Boehringer Mannheim GmbH and
used in accordance with the manufactures instructions.

10 *ANALYTICAL METHODS APPLIED TO EC-SOD VARIANTS*

*Analytical separation of EC-SOD variants from cell culture media on
Heparin-Sepharose®.*

The EC-SOD variants were separated by Heparin-Sepharose® chroma- to-
graphy using a Pharmacia FPLC apparatus, at room temperature. The
15 columns contained 1 ml Heparin-Sepharose® (Pharmacia Laboratory
Separation Division, Uppsala, Sweden) with 15 mM Na cacodylate/50 mM
NaCl, pH 6.50, as equilibration buffer and eluent. The absorbance at
280 nm was monitored on the eluent. The samples were applied at 5 ml
per hour and when the absorbance at 280 nm approached the baseline,
20 bound components were eluted with a linear gradient of NaCl in the
buffer (0-1 mol/l) at 9 ml/h. The effluent was collected in 0.65 ml
fractions and EC-SOD determined by enzyme-linked immunosorbent assay
(ELISA), which method is described below. The chloride content of
EC-SOD was determined using a standard chloride titrator (American
25 Instrument Co, Inc, Md, USA).

Before application the solvents of the culture media were exchanged
to 15 mM sodium cacodylate/50 mM NaCl, pH 6.50, on a Filtron Omega
cell membrane concentration system. In general 1-2 ml of culture
medium was applied to the Heparin-Sepharose® column.

Enzyme-linked immunosorbent assay (ELISA) for human EC-SOD

Quantitation of human EC-SOD was made using the double antibody
sandwich ELISA method. Microtiter plates (Nunclon; Nunc, Roskilde,
Denmark) were coated with 100 μl per well of a solution containing
5   16 μg/ml of polyclonal rabbit anti-EC-SOD IgG antibodies in 50 mM
Na$_2$CO$_3$, pH 9.6. After 2 h incubation at room temperature, the wells
were washed with blocking buffer (10 mM Na phosphate, pH 7.4, 140 mM
NaCl, 0.1 % wt/vol Tween 20, and 0.5 % BSA) and then blocked over-
night with 300 μl blocking buffer. For analysis, 50-μl samples,
10   diluted if necessary with blocking buffer, were added to each well
and incubated for 2 h. The wells were then washed with blocking
buffer, and 50 μl 3 μg/ml monoclonal anti-EC-SOD antibody B6,H6 which
is described in, and produced according to, Example 15 of WO 87/01387
and 50 μl peroxidase-conjugated rabbit anti-mouse IgG (DAKOPATTS,
15   Copenhagen, Denmark), both dissolved in blocking buffer, added in
that order. After a further 2 h, the wells were washed with blocking
buffer and then developed for 10 min with 100 μl 3.7 mM O-phenylene-
diamine and 0.4 mM H$_2$O$_2$ in 100 mM Na citrate, pH 5.0. After addition
of 100 μl 0.5 M H$_2$SO$_4$, the absorbance at 492 nm was determined in an
20   ELISA processor II (Hoechst Behring, Marburg, FRG). The assay was
standardized with human umbilical cord EC-SOD C. EC-SOD concentra-
tions down to ~0.25 ng/ml could be determined.

*Determination of apparent molecular weights of EC-SOD variants by
means of gel chromatography*

25   Apparent molecular weight of EC-SOD variants was determined by gel
chromatography, at 4°C using a LKB HPLC apparatus (Pharmacia LKB
Biotechnology Inc.). Culture media (1-3 ml) containing human EC-SOD
variants were applied to a Sepharyl S-300 column (Pharmacia LKB Bio-
technology Inc.) (1.6 x 89 cm), equilibrated and eluted with 10 mM
30   potassium phosphate, pH 7.4, containing 0.15 M NaCl, at a flow rate
of 19.8 ml per hour. The effluent was collected in 1.35 ml fractions
and the EC-SOD content determined by ELISA as described above. The
column was calibrated with IgG 156 (kDa) (Sigma Chemical Co., St.
Louis, USA), bovine serum albumin (67 kDa) (Sigma Chemical Co., St.

Louis, USA) and carbonic anhydrase (29 kDa) (Sigma Chemical Co., St. Louis, USA). The calibration curve was constructed by means of plotting the log molecular weights of the calibrators versus their elution volumes. Recombinant EC-SOD C and the variants T216 and T213 displayed apparent molecular weights of around 150 kDa. The glycosylation variant G1 eluted at a position corresponding to 95.5 kDa.

Analysis of superoxide dismutase activity

The superoxide dismutase activity of samples was determined by a direct spectrophotometric method employing KO_2 (S. Marklund, *J. Biol. Chem. 251*, 1976, pp. 7504-7507), as slightly modified in (S. Marklund, *In: Handbook of Methods for Oxygen Radical Research, ed; R. Greenwald, CRC press*, 1985, pp. 249-255). In brief, the basis of the method is that SOD is determined in terms of its ability to catalyze the disproportionation (decay) of $O_2 \cdot^-$ in alkaline aqueous solution. .t alkaline pH and low O_2^- concentration, the $O_2 \cdot^-$ radical is stable enough to be studied in common UV-vis spectrophotometers, using the broad absorbance maximum at 245-250 nm. Thus the assay was conducted at pH 9.5 and the disproportionation studied directly in a spectrophotometer. One unit in the assay is defined as the activity that brings about a disproportionation of $O_2 \cdot^-$ at a rate of 0.1 sec-1 in 3 ml of buffer. Under these conditions, one unit of enzyme corresponds to 8.6 ng native or recombinant human EC-SOD C (L. Tibell et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp. 6634-6638).

This assay is 40 times more sensitive (i.e. the units correspond to 40 times less enzyme) than the original xanthine oxidase - cytochrome C assay (J. M. McCord and I, Fridovich. J. Biol. Chem. 244, 1969, pp. 6049-6055).

EXAMPLE 1

*Construction of novel EC-SOD variants by site-specific mutagenesis of
human EC-SOD type C*

Nine novel EC-SOD variants were prepared by site-specific mutagenesis
5   (vide infra) of the human EC-SOD cDNA encoding EC-SOD type C obtained
from a human placenta cDNA library (K. Hjalmarsson et al., *Proc.
Natl. Acad. Sci. USA. 84*, 1987, pp. 6340-6344). The nucleotide
sequence of the cDNA encoding EC-SOD C is shown in Fig. 1. The gene-
ral strategy employed for the construction of the novel EC-SOD vari-
10   ants was to create amino acid substitutions by mutation of specific
codons, e.g. to mutate a potential glycosylation site, or to introdu-
ce translational stop codons at different positions in the cDNA or a
combination thereof.

The oligonucleotide-directed *in vitro* mutagenesis was performed using
15   a commercially available kit (Amersham, U.K.) and following the
protocol outlined below and given in detail on pages 15-47 of the
Amersham handbook "*Oligonucleotide-directed in vitro mutagenesis
system)*". This protocol, which is based on the methods of Eckstein
et. al. (J. W Taylor et. al. *Nucl. Acids Res. 13*, 1985, pp. 8749-
20   8764; J. W Taylor et. al. *Nucl. Acids Res. 13*, 1985, pp. 8764-8785;
K. Nakamaye and F. Ekstein, *Nucl. Acids Res. 14*, 1986 pp. 9679-9698)
has the advantage over other mutagenesis systems that it involves a
strand-specific selection step which eliminates the unwanted non-
mutant sequence *in vivo*, generating a pure homoduplex mutant DNA se-
25   quence. In brief, the mutagenic oligonucleotide sequence is annealed
(70°C for 3 min, 37°C for 30 min) to the single-stranded template and
extended (15 hrs at 16°C) by Klenow polymerase (12 units) in the pre-
sence of T4 DNA ligase (12 units) to generate a mutant heteroduplex.
Selective removal of the non-mutant strand is made possible by the
30   incorporation of a thionucleotide into the mutant strand during *in
vitro* synthesis. Digestion with restriction enzyme *NciI* (5 units,
37°C for 90 min) presents sites for exonuclease III (50 units *ExoIII*,
37°C for 30 min) to digest away all of the non-mutant strand of the
cloned target sequence while leaving the mutant strand intact, thus
35   enabling the mutant strand to repolymerize (3 units DNA *PolI*, 2 units

T4 DNA ligase, 16°C for 3 hrs) a homoduplex mutant DNA sequence. In
applying the method in the present example, the 1396 bp long cDNA
encoding human EC-SOD type C was cloned into the Eco R1 site of phage
M13 mp8 (J Messing and J. Vieira, Gene 19, 1982, pp 269-276) and

5 single stranded DNA template prepared in the conventional manner, as
described in detail in the Amersham handbook, pp 26-27. The template
was subsequently used together with variant oligonucleotides in the
in vitro mutagenesis reaction which was carried in the manner mentio-
ned above and described in detail in the Amersham handbook.

10 Nine different mutant oligonucleotides (21 to 23 nucleotides in
length), as illustrated in Fig. 3, were used in nine different
in vitro mutagenesis reactions to produce nine different EC-SOD
variants. The mutations introduced are outlined below.

Variant	Mutated codon	Mutated amino acid	Plasmid
T216	772 AGC → 772 TGA	217 Ser → stop	pPST216
T215	769 GAG → 769 TAG	216 Glu → stop	pPST215
T213	763 CGG → 763 TGA	214 Arg → stop	pPST213
T209	751 CGC → 751 TGA	210 Arg → stop	pPST209
SA216	769 GAG → 769 GCG	216 Glu → Ala	pPSSA216
SA219	778 TGC → 778 GCC	219 Cys → Ala	pPSSA219
SA220	781 AAG → 781 GCG	220 Lys → Ala	pPSSA220
SAT216	769 GAG → 769 GCG	216 Glu → Ala and	
	772 AGC → 772 TGA	217 Ser → stop	pPSSAT216
G1	390 AAC → 390 CAA	89 Asn → Gln	pPSG1

The mutant progenies in these reactions were identified by DNA
sequencing of single stranded DNA templates prepared from phages
produced in these *in vitro* mutagenesis reactions using the dideoxy

30 technique described in (P.H. Schreir and R. Cortese, *J. Mol. Biol.*
129, 1979, pp 169-172). On average one mutant progeny was identified
out of six sequenced templates. Double stranded replicative forms of
the mutated progenies were prepared as described in (M. Ausobel,
Current Protocols in Molecular Biology, 1, 1987, 1.15.2, digested

35 with Eco R1, and the different mutant cDNA fragments were isolated

from agarose gels by electroelution, phenol and chloroform extraction
and ethanol precipitation (Maniatis et al., *Molecular Cloning, A
Laboratory Manual, Cold Spring Harbor Laboratories, 1982*). These
mutant Eco R1 cDNA fragments were introduced into EcoR1 digested,

5   alkaline phosphatase treated plasmid pPS3, the structure of which is
shown in Fig. 2 (L. Tibell et al., *Proc. Natl. Acad. Sci. USA. 84*,
1987, pp. 6634-6638) by replacement of the wildtype cDNA Eco R1
fragment. The absence of the wildtype fragment and the presence of
the mutant EC-SOD cDNA fragments was confirmed by DNA sequencing of

10   the respective plasmid using double stranded DNA as template as
described in (O. Karlsson, *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp.
8819-8823).

Nine pPS3 derivatives were obtained which were denoted pPST216,
pPST215, pPST213, pPST209, pPSSA216, pPSSA219, pPSSA220, pPSSAT216

15   and pPSG1, respectively, and which encode the variants T216, T215,
T213, T209, SA216, SA219, SA220, SAT216 and G1, respectively. The
nucleotide sequences for each of the variants appear from Fig. 1.
Cell lines CHO DXB-11/pEE6-pSV2Ddhfr and CHO DXB-11/pEE7-pSV2dhfr
harbouring plasmids pPST216 and pPST213, respectively, have been

20   deposited on September 14, 1989 in the European Collection of Animal
Cell Cultures under the accesion numbers ECACC 890914102 and ECACC
89091403, respectively, in accordance with the provisions of the
Budapest Treaty.

In a similar manner to the one described above, i.e. using site-

25   directed mutagenesis, other EC-SOD variants having a reduced affinity
for heparin compared with native EC-SOD C may be prepared. Thus, it
is contemplated that substitution of other positively charged amino
acids besides Lys-220, especially one or more of the Lys and Arg in
the C-terminal part of EC-SOD, with a neutral or negatively charged

30   amino acid may result in a EC-SOD variant with a slightly reduced
affinity for heparin compared to EC-SOD C, which variant has retained
the superoxide dismutating property of native EC-SOD.

An EC-SOD variant having increased affinity for heparin compared to
native EC-SOD C and having retained the superoxide dismutating pro-

35   perty of native EC-SOD has been prepared by substituting the negati-

vely charged amino acid Glu in position 216 with the neutral amino
acid Ala (variant SA216). In a similar manner, other EC-SOD variants
are contemplated, which variants may be prepared from native EC-SOD C
by substitution of neutral and/or negatively charged amino acids in
5   the C-terminal part of EC-SOD C with positively charged amino acids
using site-directed mutagenesis in the same manner as described
above. Furthermore, insertion of additional basic amino acids into
the native C-terminal part of EC-SOD using synthetic DNA encoding
combinations of the amino acids arginine, lysine and histidine by
10   site-directed mutagenisis is contemplated to result in EC-SOD vari-
ants having increased affinity for heparin compared to native EC--
SOD C and having the superoxide dismutating property of native EC--
SOD.

EXAMPLE 2

15   *Expression of EC-SOD variants in CHO cells*

2.1. 20 μg of the nine pPS3 derivatives constructed in Example 1,
denoted pPST216, pPST215, pPST213, pPST209, pPSSA216, pPSSA219,
pPSSA220 and pPSG1, respectively, were transferred into dihydrofolate
reductase (dhfr) deficient CHO DXB 11 cells (G. Urlaub and L. A.
20   Chasin, *Proc. Natl. Acad. Sci. USA. 77*, 1980, pp. 4216-4220) using
the calcium phosphate coprecipitation technique as described in (F.
Graham and A. van der Eb, *Virology 52*, 1973, pp. 456-457). The cells
were grown on solid support in α + MEM medium (Gibco) supplemented
with 10% fetal calf serum (Gibco), streptomycin and penicillin.
25   Conditioned medium was harvested after three days following transfec-
tion and the presence of EC-SOD variants in the medium was confirmed
by ELISA and enzymatic activity measurements using the methods de-
scribed above.

2.2. Plasmids pPS3 and pPST213, 10 μg of each, were mixed and co-
30   transfected and the conditioned medium harvested three days following
transfection in the same manner as described in Example 2.1. above
and the distribution of EC-SOD variant proteins was analyzed using
heparin-Sepharose® chromatography as described in Example 5 below.

2.3. Two of the plasmids pPST216 and pPST213 (20 μg) carrying cDNA
encoding variants T216 and T213, respectively, were separately mixed
and cotransfected with plasmid pSV2 dhfr (0.5 μg (S.Subramani et al.,
Mol. Cell. Biol. 1, 1981, pp. 854-864) expressing dihydrofolate
5   reductase. Stable transformants were selected in selective α-MEM
medium lacking nucleosides (Gibco) supplemented with 10% dialyzed
fetal calf serum (Gibco), streptomycin and penicillin. A number of
individual transformants were isolated and expanded in the same
medium and shown by ELISA and enzymatic measurements to secrete
10   EC-SOD variants T216 and T213, respectively. For purification of
variants T216 and T213 one of each transformant was expanded into ten
1,750 cm roller bottles in 50% α-MEM 50% Optimem medium (Gibco) sup-
plemented with 0,5% newborn calf serum (Gibco), streptomycin and
penicillin. The conditioned medium was changed every third day and
15   the harvested medium was used in the purification of the EC-SOD
variants as described in Example 6 and 8. As stated above, cell lines
CHO DXB-11/pEE6-pSV2Ddhfr and CHO DXB-11/pEE7-pSV2dhfr harbouring
plasmids pPST216 and pPST213, respectively, have been deposited on
September 14, 1989 in the European Collection of Animal Cell Cultures
20   under the accesion numbers ECACC 89091402 and ECACC 89091403, respec-
tively.

The results of the analytical separation of the various preparations
are illustrated by the following figures which are further described
in Examples 4 and 5 below.

25   Fig. 4   variant T216
     Fig. 5   variant T215
     Fig. 6   variant T213
     Fig. 7   variant T209
     Fig. 8A   variant SA216
30   Fig. 8B   variant SA219
     Fig. 8C   variant SA220
     Fig. 8D   variant G1
     Fig. 9   Cotransfection of pPS3 and pPST213
     Fig. 10   Cotransfection of pPST216 and pPST213

Fig. 11   Recombinant EC-SOD C and variant T213 incubated together
           for 23 hrs.

EXAMPLE 3

Plasma clearance of recombinant EC-SOD C in rabbits

5   For purposes of comparison with the EC-SOD variants, the plasma
    clearance of recombinant EC-SOD C (L. Tibell et al., *Proc. Natl.
    Acad. Sci. USA.* 84, 1987, pp. 6634-6638) was performed. The recom-
    binant EC-SOD C was expressed from the cell line CHO-K1/pPS3neo-18
    which is available from the European Collection of Animal Cell Cul-
10 tures under the Accesion Number ECACC 86082701. The rabbits used
    were destination-bred Chinchilla ram rabbits of both sexes, age 5-6
    months and weighing 3-6 kg. Each rabbit was used for only one experi-
    ment.

    500 μg/kg body weight of the recombinant EC-SOD C dissolved in 50 mM
15 K phosphate, pH 7.4, containing 0.2 % bovine serum albumin (BSA),
    was injected into ear veins. Blood samples were tapped at times indi-
    cated in Fig. 12 into tubes containing EDTA as anticoagulant. Final-
    ly, 2,500 IU/kg body wt heparin was injected, and blood samples were
    tapped at 1, 5, and 15 min thereafter. After centrifugation at
20 2,000 g for 10 min, 1 ml plasma was collected and the human EC-SOD
    content was determined by ELISA in the Analytical Methods section
    above.

    Plasma volume. In each animal, the plasma volume was determined by
    means of injecting, together with the recombinant EC-SOD C, a known
25 amount of 125 I-labelled human serum albumin. The radioactivity of
    collected blood samples was determined by γ-ray detection. The counts
    were extrapolated to time zero for calculation of the plasma volume.

    The results are shown in Fig. 12. It is seen that after intravenous
    injection, recombinant EC-SOD C is rapidly cleared from plasma, down
30 to about 2-3% of the expected level (if all enzyme remained in plas-
    ma). If a large dose of heparin is injected after 20 min all seque-

stered (up to 100%) enzyme is released to plasma. Later injections,
at 300 min and 24 hrs, result in less recovery of bound enzyme, but
more than 30% is still released at 24 h. Available data (K. Karlsson
and S. L. Marklund, *Biochem. J. 242*, 1987, pp. 55-59; K. Karlsson and
5   S. L. Marklund, *J. Clin. Invest. 82*, 1988, pp. 762-766; K. Karlsson
et al., *Biochem. J. 256*, 1988, pp. 29-33; S. L. Marklund and K.
Karlsson, *Lab. Invest. 60*, pp. 695-666, 1989) indicate that the
injected EC-SOD C is bound by heparan sulfate proteoglycan in the
glycocalyx of vessel endothelium. When heparin is injected, the
10 enzyme instead binds to the heparin, which has a higher affinity for
EC-SOD C than heparan sulfate (K. Karlsson et al., *Biochem. J. 256*,
1988, pp. 29-33).

EXAMPLE 4

Analysis of the EC-SOD variants prepared in Examples 1 and 2

15 *4.1. Variant T216*

Variant T216 was produced as described in Examples 1 and 2. When
separated on Heparin-Sepharose® as described in the analytical method
section above this variant eluted at about 0.42 M NaCl (Fig. 4) and
has thus a lower affinity than recombinant and native EC-SOD C which
20 elutes at about 0.55 M NaCl (L. Tibell et al., *Proc. Natl. Acad. Sci.
USA. 84*, 1987, pp. 6634-6638). This variant thus belongs to C-class
EC-SOD. Its molecular weight as deduced from gel chromatography was
like recombinant EC-SOD C approximately 150 kDa and it is thus likely
to be tetrameric. In this variant one positively charged amino acid,
25 Lys 220, one negatively charged Glu 218, and 4 neutral amino acids
were lost, a modification which resulted in a slightly reduced
heparin affinity.

When intravenously injected into rabbits in the manner described for
recombinant EC-SOD C in Example 3, variant T216, like EC-SOD C, is
30 rapidly sequestered from the plasma, Fig. 13. However, considerably
more, 20%-30%, remains in the plasma phase. Injection of heparin at
25 min, 120 min, 300 min and 24 h, resulted in a prompt release of

variant T216 to plasma, indicating binding of the variant EC-SOD to
heparan sulfate on vessel endothelium. However, in accordance with
the reduced heparin affinity, as compared with that of native and
recombinant EC-SOD C, the equilibrium between plasma and endothelium
5   was considerably displaced towards the plasma phase, c.f. Figs. 12
and 13.

4.2. *Variant T215*

Variant T215 was produced as described in Examples 1 and 2. Cell
culture medium separated on Heparin-Sepharose® as described in the
10   analytical methods section above is illustrated in Fig. 5 which shows
that this variant lacks affinity for Heparin-Sepharose® and belongs
to EC-SOD of class A. The total loss of heparin affinity was unexpec-
ted in view of the fact that only one negatively charged amino acid,
Glu 216, had been removed. Further removal of amino acid residues as
15   in variants T213 and T209 below, also resulted in A-class EC-SODs.

The presence of the part of the cluster with the highest charge-
density, Arg-210, Lys-211, Lys-212, Arg-213, Arg-214, Arg-215, is
thus suprisingly not sufficient for any heparin affinity.

4.3. *Variant T213*

20   Variant T213 was produced as described in Examples 1 and 2. Cell
culture medium separated on Heparin-Sepharose® as described in the
analytical method section above (Fig. 6) shows that this variant
lacks affinity for heparin and thus belongs to EC-SOD class A. Its
molecular weight as determined from gel chromatography was like
25   recombinant EC-SOD C approximately 150 kDa and is thus likely to be
tetrameric.

When variant T213 was intravenously injected into rabbits, apparently
all enzyme remained in the plasma phase, Fig. 14. Injection of hepa-
rin at 300 min and 24 h, led to no significant release of this vari-
30   ant enzyme i.e. there was no evidence for binding of A-class EC-SOD
to vessel endothelium.

4.4. *Variant T209*

Variant T209 was produced as described in Examples 1 and 2. When medium containing the variant is separated on Heparin-Sepharose® as described in the analytical method section above (Fig. 7), it is
5   found that this variant lacks affinity for heparin and thus belongs to EC-SOD class A.

4.5. *Variant SA216*

Variant SA216 was produced as described in Examples 1 and 2. Cell culture medium separated on Heparin-Sepharose® as described in the
10 analytical method section above (Fig. 8A) shows a peak eluting at 0.6 M NaCl and thus has a higher affinity for heparin than recombinant and native EC-SOD C. In this variant one negatively charged amino acid, Glu 216, has been substituted with one neutral amino acid, Ala.

15 ### 4.6. *Variant SA219*

Variant SA219 was produced as described in Examples 1 and 2. Cell culture medium separated on Heparin-Sepharose® as described in the analytical method section above (Fig. 8B) shows a peak eluting at about 0.55 M NaCl and thus has the same affinity as recombinant and
20 native EC-SOD C. In this variant one cystein residue, Cys 219, has been substituted with one Ala. This excludes the possibility that Cys 219 is engaged in a S-S bridge that is involved in maintaining the correct structure of the C-terminal to obtain heparin affinity. The fact that the variant SA219 has the same affinity as recombinant
25 EC-SOD C illustrates that the substitution of a neutral amino acid (Cys) with another neutral amino acid (Ala) apparently has no effect on the heparin binding affinity.

4.7. *Variant SA220*

Variant SA220 was produced as described in Examples 1 and 2. Cell
30 culture medium separated on Heparin-Sepharose® as described in the

analytical method section above (Fig. 8C) shows a peak eluting at
0.5 M NaCl and thus has a slightly reduced affinity for heparin. In
this variant one positively charged amino acid, Lys 220, has been
substituted with one neutral amino acid, Ala. Thus, it is illustrated
5   that the heparin binding affinity remains unchanged when the net
charge of this variant is unchanged.

4.8 *Variant SAT216*

Variant SAT216 was produced as described in Examples 1 and 2. Cell
culture medium separated on Heparin-Sepharose® as described in the
10 analytical method section above (Fig. 8E) shows a peak eluting at
0.51 M NaCl and thus has a lower affinity than recombinant and native
EC-SOD C. In this variant amino acid Glu 216 has been substituted
with the amino acid Ala and the rest of the C-terminal has been trun-
cated. This variant has the same amino acid chain length as variant
15 T216, but the negatively charged amino acid Glu 216 has been substi-
tuted with a neutral amino acid Ala, thus taking away one negative
charge from the C-terminal, and hence giving it a higher net positive
charge and a higher affinity for heparin than variant T216.

Variant T215 which contains the part of the amino acid cluster with
20 the highest charge density, Arg 210, Lys 211, Lys 212, Arg 213, Arg
214, Arg 215, completely lacks heparin affinity, whereas variants
T216 and SAT216 with Glu and Ala, respectively, in position 216 both
being only one amino acid longer, both have high heparin affinity.
Surprisingly, the high positive charge density cluster is not suffi-
25 cient for a high heparin affinity. It appears that still one more
amino acid residue is needed to obtain a conformation resulting in
heparin affinity.

4.9. *Variant G1*

Variant G1, a glycosylation-free variant, was produced as described
30 in Examples 1 and 2. Analytical separation on Heparin-Sepharose® as
described in the analytical method section above (Fig. 8D) shows two
peaks, one eluting at 0.38 M NaCl and one with very high heparin
affinity which elutes at 0.64 M NaCl. Its molecular weight as deter-

mined from gel chromatography performed as described in the analy-
tical method section above was 95.5 kDa, very similar to the figure
expected for a tetramer with a subunit molecular weight of 24.2 kDa
subunit, the molecular weight calculated from the EC-SOD C amino acid

5   content.

The absence of carbohydrate in this variant was demonstrated by the
lack of binding to the lectin concanavalin A immobilised on Sepha-
rose®. Culture medium containing variant G1 was passed through a Con
A-Sepharose® column (Pharmacia LKB Biotechnology Inc.) as previously

10   described for native and recombinant EC-SOD C (L. Tibell et al.,
Proc. Natl. Acad. Sci. USA. 84, 1987, pp. 6634-6638). In brief,
following appplication of the culture medium, the Con A-Sepharose®
column was eluted first with sodium phosphate buffer, and then, in
order to free any bound EC-SOD, with 0.5 M methyl α-mannoside. The

15   degree of binding to concanavalin A was calculated from the SOD
activity (determined as described in the Analytical Methods section)
of the eluates. In contrast to EC-SOD C, the EC-SOD-ELISA reactive
material of the variant G1-containing culture medium did not bind to
the lectin concanavalin A.

20   At present there is no explanation for the heterogeneity in heparin
affinity. An increased heparin affinity may be due to the fact the
carbohydrate substituent of EC-SOD carries a considerable negative
charge that may repel the enzyme from the strongly negatively charged
heparin. A removal of this effect may increase the heparin affinity.

25   *EXAMPLE 5*

Analysis of the heterotetramers of EC-SOD produced in Example 2

5.1. Cotransfection of plasmid pPST213 and pPS3

Plasmids pPST216 and pPS3 were cotransfected as described in Example
2.3 above. The resulting culture medium was separated on Heparin-

30   Sepharose® as described in the analytical method section above (Fig.
9). The chromatogram contained one non-binding peak of class A (simi-

lar to what is obtained when pPST213 is transfected alone) and one
strongly binding peak eluting at about 0.52 M NaCl (similar to the
peak obtained upon transfection of only pPS3, that is recombinant
EC-SOD C). In addition two weaker binding peaks eluting at 0.18 and
5   0.28 M NaCl are seen. These would by definition belong to B-class
EC-SOD.

5.2. *Cotransfection of plasmid pPST216 and pPST213*

Plasmid pPST216 and pPST213 were cotransfected as described in Exam-
ple 2.2 above. The resulting culture medium was separated on Heparin-
10   Sepharose® as described in the analytical method section above (Fig.
10). The chromatogram contained one non-binding peak of class A
(similar to what was obtained when pPST213 was transfected alone) and
one relatively strongly binding peak eluting at about 0.40 M NaCl
(similar to the peak obtained upon transfection of only pPST216). In
15   addition two weaker binding peaks eluting at 0.13 and 0.23 M NaCl are
seen. These would by definition belong to B-class EC-SOD.

5.3. *Coincubation of recombinant EC-SOD C and variant T213*

Culture media containing variant T213 (prepared as described above,
Example 2) and recombinant EC-SOD C (L. Tibell et al., *Proc. Natl.*
20   *Acad. Sci. USA.* 84, 1987, pp. 6634-6638) were mixed (about 100 ng/ml
in α-medium containing 10% fetal calf serum (Gibco)) so as to produce
a solution containing initially nearly equal amounts of each variant.
The mixture was incubated at 37°C. Analytical separation of the mix-
ture (after different incubation times) on Heparin-Sepharose® as
25   described in the Analytical Methods section above resulted in the
pattern seen in Fig. 11 which illustrates samples taken shortly after
mixing and after 23 hrs of coincubation. One non-binding class A
peak is seen (corresponding to that which is obtained when variant
T213 is analyzed, Fig. 6) and one strongly binding peak eluting at
30   0.40 M NaCl (similar to what is obtained when recombinant EC-SOD C
alone is analyzed). In addition a broad peak eluting at 0.28 M NaCl
is seen, belonging by definition to class B EC-SOD.

Conclusion

Cotransfection of pPST213/pPST216 and pPST213/pPS3 thus resulted, in
addition to formation of the parental types, in the formation of two
intermediate B-class forms. In the case of pPST213/pPST216 the hepa-
5   rin affinities of the B-forms were a little lower than in the case
of pPST213/pPS3. This is in accord with the lower heparin affinity of
variant T216 compared with recombinant EC-SOD C. In the coincubation
experiment with variant T213 and recombinant EC-SOD C, a B-class
intermediate was also gradually formed. This intermediate had a hepa-
10   rin affinity equal to that of the stronger binding B-class inter-
mediate of the cotransfection experiment with the corresponding plas-
mid combination. Taken together these results strongly suggest that
B-class EC-SODs are heterotetramers of A- and C-class monomers. The
most likely compositions are the following (small letters denote
15   subunits):

cccc for C-class;
ccca and ccaa for the two B-class peaks respectively; and
caaa and aaaa for A-class EC-SOD.

Example 6

20   *Purification of EC-SOD variant T216*

Variant T216 was prepared as described in Examples 1 and 2. The
resulting transfected cells were expanded and finally 2,930 ml cul-
ture medium containing 748 units SOD activity and 4,750 μg EC-SOD
(ELISA) per ml was obtained.

25   6.1.   *Separation on Q-Sepharose®*
The culture medium was applied to a Q-Sepharose® column (Pharmacia
LKB Biotechnology Inc.), 104 ml, equilibrated with 50 mM K phosphate,
pH 7.6. The medium was applied at 360 ml/h and the column was then
washed with 200 ml equilibration buffer at the same rate. Bound
30   enzyme was then eluted at 360 ml/h with 400 mM K phosphate, pH 7.6

(Fig. 15). Eluted fractions were analyzed for SOD activity. The peak
of SOD activity was pooled as indicated in the figure.

6.2.   *Separation on Phenyl-Sepharose®*

The pool from the Q-Sepharose® step was dialyzed against 600 mM Na
phosphate, pH 7.6, and was then applied at 165 ml/h to a Phenyl-
Sepharose® column (Pharmacia LKB Biotechnology Inc.), 10 ml, equi-
librated with 600 mM Na phosphate. The column was then washed with
125 ml equilibration buffer. Bound enzyme was finally eluted with
10 mM Na phosphate, pH 7.6, as seen in Fig. 16. The enzyme was pooled
as indicated in the figure.

6.3.   *Separation on Heparin-Sepharose®*

The pool from the Phenyl-Sepharose® step was dialyzed against 0.1 M
Na phosphate, pH 7.6, and then applied at 45 ml/h on a Heparin-Sepha-
rose® column, 10 ml equilibrated with 0.1 M Na phosphate, pH 7.6. The
column was then washed with 33 ml equilibration buffer. Bound enzyme
was finally eluted with a gradient in NaCl (0-1 M) in the equilibra-
tion buffer and the SOD activity was determined in collected frac-
tions as described in the analytical method section above. Eluted
enzyme was pooled as indicated in Fig. 17 and finally concentrated.

Example 7

Analyses on isolated EC-SOD variant T216

The results of the purification steps performed in Example 6 are
summarized in Table X.

The final specific activity (units SOD activity per ml/absorbance at
280) 67,000 is similar to that previously obtained for recombinant
EC-SOD C, 69,400 (L. Tibell et al., *Proc. Natl. Acad. Sci. USA. 84,*
1987, pp. 6634-6638).

The purity of the isolated EC-SOD variant T216 was determined by
means of polyacrylamide gel electrophoresis in the presence of sodium
dodecylsulfate, as described in the legend to Fig. 18, and the iden-

tity as a human EC-SOD by means of Western blotting, as described in
the legend to Fig. 19. No impurity could be detected by the poly-
acrylamide gel electrophoresis. Partially purified variant T213, as
obtained in Example 8, was also electrophoresed (Fig. 18) and its
5   identity determined by Western blotting (Fig. 19). As can be seen,
there is a distinct decline in apparent molecular weights going from
recombinant EC-SOD C, to variant T216 and to variant T213. This is in
accord with the decreasing molecular weights resulting from the
carboxyterminal truncations.

10   Table X.   *Isolation of EC-SOD variant T216*

Results after each separation step are shown. The SOD activity was
determined by means the direct spectrophotometric method employing
KO_2 (S. Marklund, J. Biol. Chem. 251, 1976, pp. 7504-7507). One unit
corresponds to 8.6 ng native and recombinant human EC-SOD C (L.
15   Tibell et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp. 6634-6638).

	SOD activity (units)	Specific activity (units per ml/A_280)	Recovery %
Culture medium	1,328,000	-	100 %
1. Q-Sepharose®	1,091,000	10,555	82.1 %
2. Phenyl-Sepharose®	969,000	21,440	73 %
3. Heparin-Sepharose®	553,700	67,034	41.7%

Example 8

25   *Partial purification of EC-SOD variant T213*

Variant T213 was prepared as described in Examples 1 and 2. The
resulting transfected cells were expanded and finally 860 ml culture
containing 162.5 units SOD activity per ml was obtained.

8.1. *Anti EC-SOD-Sepharose®*

Monoclonal anti-human EC-SOD antibody 14-B7 (which is described in, and produced according to, Example 15 of WO 87/01387) was coupled to CNBr-activated Sepharose® 4B (Pharmacia LKB Biotechnology Inc.) at a
5   concentration of 2 mg/ml gel. The gel, 28 ml, was packed in a chromatography column and equilibrated with 50 mM 1-aminomethyl-propanol hydrochloride pH 9.0/0.5 M NaCl. The culture medium was applied at 100 ml/h. Unexpectedly no binding of the EC-SOD variant occurred. This monoclonal antibody had previously successfully been employed
10   for the isolation of native and recombinant EC-SOD C (L. Tibell et al., *Proc. Natl. Acad. Sci. USA.* 84, 1987, pp. 6634-6638). The affinity of the various EC-SOD variants for the immobilized antibody was subsequently tested. It was found that variants T213 and T209, which lack binding to heparin and thus belong to the A-class, also
15   lack affinity for the antibody. On the other hand native and recombinant EC-SOD C and variant T216, which belongs to the C-class EC-SOD were all bound by the antibody. This monoclonal antibody, 14-B7, thus has its specificity directed towards a functional heparin-binding site of EC-SODs.

20   ### 8.2. *Separation on Q-Sepharose®*

After direct passage through the anti EC-SOD-Sepharose® column the medium containing variant T213 (875 ml, 145.6 SOD units per ml) was applied to a Q-Sepharose® column (Pharmacia LKB Biotechnology Inc.), 40 ml, equilibrated against 50 mM K phosphate pH 7.6 at 300 ml/h. The
25   column was then washed with equilibration buffer. Bound enzyme was then eluted with a gradient in K phosphate, pH 6.0, 50-300 mM, Fig. 20.

The SOD activity was determined in collected fractions and the peak was pooled in two fractions, I and II.

30   ### 8.3. *Chromatography on Heparin-Sepharose®*

Pool I (139 ml) was concentrated to 12 ml and dialyzed against 25 mM K phosphate, pH 6.5. It was then applied to a Heparin-Sepharose® column, 5 ml, equilibrated against 25 mM K phosphate, pH 6.5. The SOD activity eluted without binding to the column, and thus represented
35   EC-SOD of class A. The eluted enzyme was pooled, the total SOD acti-

vity of which was 43,000 units. According to ELISA as described in
the analytical method section above the EC-SOD content was 740 μg.
One unit thus corresponded to 17 ng EC-SOD protein, a figure not far
away from the one previously obtained for native and recombinant EC-

5   SOD C (L. Tibell et al., *Proc. Natl. Acad. Sci. USA. 84*, 1987, pp.
6634-6638). The variant T213 could easily be detected upon electro-
phoresis in polyacrylamide gel in the presence of sodium dodecylsul-
fate as described in the legend to Fig. 18. The identity of the pro-
tein as EC-SOD was established by means of Western blotting, as de-

10 scribed in the legend to Fig. 19.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page ____21____, line ____24____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

European Collection of Animal Cell Cultures
(ECACC)

Address of depositary institution (including postal code and country) [4]
Porton Down
Salisbury, Wiltshire SP4 0JG
Great Britain

Date of deposit [5]	Accession Number [6]
14 September, 1989	ECACC 89091402

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

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MICROORGANISMS

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A. IDENTIFICATION OF DEPOSIT [2]

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Date of deposit [5]	Accession Number [6]
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deposited microorganisms only be made available to an expert
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is granted or the date on which the application has been re-
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CLAIMS

1. A polypeptide having the superoxide dismutating property of the
native extracellular superoxide dismutase (EC-SOD) and having a
modified binding to heparin as compared with recombinant EC-SOD C,

5   the polypeptide having the formula

A-R

wherein A designates the EC-SOD C amino acid sequence from and in-
cluding amino acid moiety No. 1 up to and including amino acid moiety
No. 193 (*vide* Fig. 1A or Fig. 1B)

10   wherein R is an amino acid sequence conferring to the polypep-
        tide either

        a decreased affinity for heparin compared to recombinant
        EC-SOD C as a result of the sequence

15   being truncated from its C-terminal end compared to
                the amino acid sequence constituting amino acid moie-
                ties 194-222 of recombinant EC-SOD C,

                and/or having a smaller positive net charge than the
                net charge of the amino acid sequence constituting
                amino acid moieties 194-222 of recombinant EC-SOD C,

20   or

        an increased affinity for heparin compared to recombinant
        EC-SOD C as a result of the sequence

                having a larger positive net charge than the net
                charge of the amino acid sequence constituting amino
25   acid moieties 194-222 of recombinant EC-SOD C,

the glycosylation site of the polypeptide in the above
cases optionally being replaced by a moiety which
cannot be glycosylated, or

5   R is the amino acid sequence constituting amino acid moieties
           194-222 of recombinant EC-SOD C, in which case the glycosylation
           site of the polypeptide has been replaced with a moiety which
           cannot be glycosylated, whereby the heparin affinity of the
           polypeptide has been increased, compared with recombinant
10   EC-SOD C,

and modifications thereof which retain the superoxide dismutating
property of native EC-SOD.

2. A polypeptide according to claim 1 wherein the smaller positive
net charge of R compared to the net charge of the amino acid sequence
15 constituting amino acid moieties 194-222 of recombinant EC-SOD C has
been obtained by

           removal of one or several positively charged amino acid moieties
           compared to the amino acid sequence constituting amino acid
           moieties 194-222 of recombinant EC-SOD C

20   and/or substitution of one or several positively charged amino
           acid moieties of the amino acid sequence constituting amino acid
           moieties 194-222 of recombinant EC-SOD C with negatively charged
           and/or neutral amino acid moieties,

           and/or addition of negatively charged amino acid moieties,
25   compared to the corresponding sequence in EC-SOD C,

           and/or chemical modification of one or more amino acid moieties
           to introduce negative charges.

3. A polypeptide according to claim 2 in which R designates a trun-
cated version of the amino acid sequence constituting amino acid
30 moieties 194-222 of recombinant EC-SOD C, the C-terminal end of the

sequence having been truncated with 1-12 amino acids, such as 1-7
amino acids.

4. A polypeptide according to claim 3, wherein the C-terminal end has
been truncated with 1-6 amino acids, such as 1-5 amino acids.

5   5. A polypeptide according to claim 1, in which R comprises at least
23 amino acid moieties.

6. A polypeptide according to claim 1 wherein the larger positive net
charge of R compared to the net charge of the amino acid sequence
constituting amino acid moieties 194-222 of recombinant EC-SOD C has
10   been obtained by

        removal of negatively charged amino acid moieties compared to
        the amino acid sequence constituting amino acid moieties 194-222
        of recombinant EC-SOD C,

        and/or substitution of neutral and/or negatively charged amino
15   acid moieties with positively charged amino acid moieties,
        compared to the amino acid sequence constituting amino acid
        moieties 194-222 of recombinant EC-SOD C,

        and/or addition of positively charged amino acid moieties,
        compared to the amino acid sequence constituting amino acid
20   moieties 194-222 of recombinant EC-SOD C,

        and/or chemical modification of one or more amino acid moieties
        to introduce positive charges.

7. A polypeptide according to claim 1, wherein R contains one or
several of the following amino acid moieties:

25

Arg, Lys, Glu, Ser, Cys, and His.

8. A polypeptide, termed T216, according to claim 2 which has a
reduced binding to heparin as compared with recombinant EC-SOD C,
30   wherein

R is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-
Arg-Lys-Lys-Arg-Arg-Arg-Glu.

9. A polypeptide according to claim 2 which has a reduced binding to
heparin as compared with recombinant EC-SOD C, which polypeptide is

5 selected from the group consisting of the polypeptide termed T215,
wherein

R is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-
Arg-Lys-Lys-Arg-Arg-Arg;

the polypeptide termed T209, wherein

10 R is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu;

the polypeptide termed SA220, wherein the amino acid Lys of the
native EC-SOD C has been replaced with an Ala moiety; and

the polypeptide termed SAT216, wherein R is Val-Cys-Gly-Pro-Gly-Leu-
Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-Arg-Lys-Lys-Arg-Arg-Arg-Ala

15 and the amino acid Glu 216 has been replaced with an Ala moiety.

10. A polypeptide, termed T213, according to claim 2 which has a
reduced binding to heparin as compared with recombinant EC-SOD C,
wherein

R is Val-Cys-Gly-Pro-Gly-Leu-Trp-Glu-Arg-Gln-Ala-Arg-Glu-His-Ser-Glu-

20 Arg-Lys-Lys-Arg.

11. A polypeptide, termed SA216, according to claim 2 which has an
increased binding to heparin as compared with recombinant EC-SOD C,
wherein the amino acid Glu of the native EC-SOD C has been replaced
with an Ala moiety.

25 12. A polypeptide, termed G1, according to claim 1 which has an
increased binding to heparin as compared with recombinant EC-SOD C
and which is non-glycosylated, wherein the glycosylation site Asn 89

in the polypeptide of recombinant EC-SOD C has been replaced with a
Gln-moiety.

13. A polypeptide composition which has the superoxide dismutating
property of native EC-SOD and which comprises several polypeptides as
5   claimed in any of claims 1-12, or one or more polypeptides as claimed
in any of claims 1-12 in admixture with EC-SOD A, EC-SOD B or
EC-SOD C.

14. A polypeptide composition which has the superoxide dismutating
property of native EC-SOD and which is an oligomer comprising several
10 polypeptides as claimed in any of claims 1-12, or one or more poly-
peptides as claimed in any of claims 1-12 in admixture with EC-SOD A,
EC-SOD B or EC-SOD C.

15. A polypeptide composition according to claim 14 which is a tetra-
mer comprising four polypeptides as claimed in any of claims 1-12, or
15 one or more polypeptides as claimed in any of claims 1-12 in admix-
ture with EC-SOD A, EC-SOD B or EC-SOD C.

16. A polypeptide composition according to claim 15 wherein the
polypeptides are identical.

17. A polypeptide composition according to claim 13, the composition
20 having a lower mean heparin binding than recombinant EC-SOD C.

18. A polypeptide composition according to claim 13, 14, 15, or 17,
which is composed of a polypeptide according to claim 8 and a poly-
peptide according to claim 10.

19. A polypeptide composition according to claim 13, 14, 15 or 17,
25 which is composed of a polypeptide according to claim 8 and EC-SOD C.

20. A polypeptide or polypeptide composition according to any of
claims 1, 6, 7 or 12-15, which has an increased affinity for heparin
compared to recombinant EC-SOD C and which has a (mean where appro-
priate) heparin binding affinity corresponding to complete elution of
30 the polypeptide or polypeptide composition from a heparin-Sepharose

column with between 0.55 and 1.5 molar aqueous NaCl under conditions
as described in the Analytical Methods section herein under the
heading "Separation of EC-SOD variants from cell culture media by
Heparin-Sepharose Chromatography".

5   21. A polypeptide or polypeptide composition according to claim 20
which has a (mean where appropriate) heparin binding affinity cor-
responding to complete elution of the polypeptide or polypeptide
composition from a heparin-Sepharose column with between 0.55 and 1.0
molar aqueous NaCl, such as with between 0.55 and 0.75 molar aqueous
10   NaCl, under conditions as described in the Analytical Methods section
herein under the heading "Separation of EC-SOD variants from cell
culture media by Heparin-Sepharose Chromatography".

22. A polypeptide or polypeptide composition according to claim 21
which has a (mean where appropriate) heparin binding affinity cor-
15   responding to complete elution of the polypeptide or polypeptide
composition from a heparin-Sepharose column of between 0.55 and 0.65
molar aqueous NaCl under conditions as described in the Analytical
Methods section herein under the heading "Separation of EC-SOD vari-
ants from cell culture media by Heparin-Sepharose Chromatography".

20   23. A polypeptide or polypeptide composition according to any of
claims 1-5, 7-11 or 12-18, which has a reduced affinity for heparin
compared to recombinant EC-SOD C and which has a (mean where appro-
priate) heparin binding affinity corresponding to complete elution of
the polypeptide or polypeptide composition from a heparin-Sepharose
25   column with between 0 and 0.54 molar aqueous NaCl under conditions as
described in the Analytical Methods section herein under the heading
"Separation of EC-SOD variants from cell culture media by Heparin--
Sepharose Chromatography".

24. A polypeptide or polypeptide composition according to claim 23
30   which has a (mean where appropriate) heparin binding affinity cor-
responding to complete elution of the polypeptide or polypeptide
composition from a heparin-Sepharose column with between 0.0 and 0.50
molar aqueous NaCl, such as between 0.0 and 0.45 molar aqueous NaCl,
e.g. between 0.0 and 0.35 molar aqueous NaCl, under conditions as

described in the Analytical Methods section herein under the heading
"Separation of EC-SOD variants from cell culture media by Heparin--
Sepharose Chromatography".

25. A polypeptide or polypeptide composition according to any of
claims 1-11 and 13-24 which is glycosylated.

26. A DNA fragment encoding a polypeptide of any of claims 1-12.

27. A DNA fragment according to claim 26 which is of synthetic origin
or which is obtained by modification of a DNA sequence of genomic or
complementary DNA origin, which DNA sequence encodes native EC-SOD C.

28. A replicable expression vector comprising a DNA fragment of claim
27.

29. A cell harboring a vector according to claim 28.

30. A cell according to claim 29, which is a procaryotic or eucary-
otic cell, especially a mammalian cell.

31. A cell according to claim 30, in which is the mammalian cell
CHO DXB 11/pEE6-pSV2Ddhfr which was deposited in the European Collec-
tion of Animal Cell Cultures under the Accession number ECACC
89091402 on the September 14, 1989, or the mammalian cell line
CHO DXB 11/pEE7-pSV2dhfr which was deposited in the European Collec-
tion of Animal Cell Cultures under the Accession number ECACC
89091403 on the September 14, 1989.

32. A method of producing a polypeptide as defined in any of claims
1-12 comprising modifying a DNA sequence encoding native EC-SOD C by
a mutagenic treatment so as to obtain a DNA fragment as defined in
claim 26, inserting said DNA fragment into a vector which is able to
replicate in a host cell, introducing the resulting recombinant
vector into the host cell, culturing the host cell in an appropriate
culture medium under appropriate conditions for expressing the poly-
peptide, and recovering the polypeptide from the host cell or culture
medium.

33. A method according to claim 32, in which the DNA sequence encoding native EC-SOD C is of complementary DNA origin or genomic origin.

34. A method according to claim 33, in which the DNA sequence encoding native EC-SOD C is of mammalian origin, especially human origin.

5   35. A method according to any of claims 32-34, in which the DNA sequence is the sequence encoding EC-SOD C shown in Fig. 1.

36. A method according to any of claims 32-35, in which the DNA sequence is modified by site-specific mutagenesis.

37. A method according to claim 36, in which the site-specific muta-
10   genesis is carried out under suitable conditions on a single strand of the DNA sequence encoding EC-SOD C by use of an oligonucleotide which is identical to a subsequence of one of the strands of the DNA sequence encoding EC-SOD C except for at least one nucleotide which has been inserted, deleted, substituted or added to the sequence.

15   38. A method according to claim 37, in which the oligonucleotide differs from the corresponding part of the DNA sequence encoding EC-SOD C in that

a) it has an insert within its sequence of at least 3 nucleo-
tides such as at least 6 nucleotides which is different from the
20   corresponding part of the DNA sequence encoding EC-SOD C,

b) its sequence is at least 3 nucleotides, such as at least 6 nucleotides shorter than the corresponding part of the DNA sequence encoding EC-SOD C because of deletion of these nucleo-
tides within or at one or both of the ends of the sequence,

25

c) its sequence differ from the corresponding part of the DNA sequence encoding EC-SOD C in that at least one nucleotide of the DNA sequence encoding EC-SOD C has been substituted with another nucleotide, and/or

d) at least one nucleotide has been added to one or both of the
ends of the DNA sequence encoding EC-SOD C.

39. A method according to claim 37 or 38, in which the oligonucleo-
tide comprises 5-150 nucleotides, preferably 5-50, more preferably
5   7-30, still more preferably 7-25 and most preferable about 21 or 23
nucleotides.

40. A method according to any of claims 37-39, in which the oligonu-
cleotide is of synthetic origin.

41. A method according to any of claims 36-40, in which the single
10   strand of the DNA sequence encoding EC-SOD C has been obtained by
cloning in the vector M13.

42. An oligonucleotide which is substantially identical to at least a
subsequence of one of the strands of the DNA sequence encoding
EC-SOD C except for the presence or absence of at least one nucleo-
15   tide different from the nucleotide at the same position of the DNA
sequence encoding EC-SOD C, which oligonucleotide is suitable for use
in site-specific mutagenesis to be used in the preparation of a
polypeptide as claimed in any of claims 1-12.

43. A method of preparing a polypeptide as claimed in any of
20   claims 1-12, comprising inserting a DNA sequence encoding native
EC-SOD C into an expression vector, introducing said vector into a
host cell capable of expressing EC-SOD C, growing said host cell
under condition ensuring the expression of substantial recombinant
EC-SOD C, subjecting the expressed substantial recombinant EC-SOD C
25   to a posttranslational modification so as to obtain a polypeptide as
defined in any of claims 1-12, and recovering said polypeptide.

44. A method according to claim 43, wherein the posttranslational
modification comprises chemical modification of the amino acid resi-
dues of the polypeptide sequence of recombinant EC-SOD C.

30   45. A method of preparing a polypeptide as claimed in any of claims
1-12 comprising subjecting native EC-SOD C to an enzymatic or chemi-

cal treatment, by which the native EC-SOD C is converted into a
polypeptide as defined in any of claims 1-12.

46. A method of preparing a polypeptide composition as claimed in any
of claims 13-19, comprising

5

> a) cotransfecting expression vectors which separately comprise
> the DNA sequence encoding each of the polypeptides to be in-
> cluded in the polypeptide composition by introducing each of the
> vectors into a host cell which is capable of expressing the
> polypeptides, culturing the host cell under conditions suitable
> for expressing each of the polypeptides to be included in the
> polypeptide composition, and recovering and separating the
> culture medium comprising the polypeptide composition, or

10

> b) coincubating a sample of each of the polypeptides to be
> included in the polypeptide composition under suitable condi-
> tions so as to obtain the polypeptide composition.

15

47. A method according to any of claims 32-41 or 43-46, in which the
polypeptide is recovered by absorption to a matrix containing

20

> a) immobilized antibodies against the polypeptide or an immuno-
> logical subsequence thereof, or

> b) heparin or a heparin analogue, e.g. heparan sulphate, or
> another sulphated glucosaminoglycane, dextran sulphate or
> another strongly negatively charged compound.

25 eluting the polypeptide from the matrix, pooling the eluted fractions
containing the polypeptide, and optionally subjecting the pooled
fractions to further purification.

48. A method according to claim 47, in which the polypeptide is
eluted with NaCl.

30 49. A method according to any of claims 32-41 or 43-48, in which
heparin or a heparin analogue, e.g. heparan sulphate, sulphated

glucosaminoglycane, dextran sulphate or another strongly negatively charged compound, is added to the medium in which the cells are grown in an amount sufficient to induce release of the polypeptide from the cell surfaces.

5   51. Use of a polypeptide or a polypeptide composition according to any of claims 1-24 for the prophylaxis or treatment of diseases or disorders connected with the presence or formation of superoxide radicals and other toxic oxygen intermediates derived from the super-oxide radical.

10   52. Use according to claim 51, in which the disease or disorder is selected from conditions involving ischemia followed by reperfusion, e.g. infarctions such as heart, kidney, brain or intestine infarc-tions, inflammatory diseases such as rheumatoid arthritis, pancrea-titis, in particular acute pancreatitis, pyelonephritis and other
15   types of nephritis, and hepatitis, neuritis, uveitis, cystitis, peyronies disease, autoimmune diseases, insulin-dependent diabetes mellitus, disseminated intravascular coagulation, fatty embolism, haemorrhagic shock, endotoxin-induced shock, septicemia, severe viral infections, adult respiratory distress, infantile respiratory dis-
20   tress, brain haemorrhages in neonates, burns, preservation of lens and cornea in the context of transplantation, adverse effects of ionizing radiation, and carcinogenesis, and adverse effects of toxins such as alloxan, paraquat, and some cytostatic compounds.

53. Use of a polypeptide or a polypeptide composition according to
25   any of claims 1-24 in connection with the transplantation of organs and body tissues such as kidney, lung, heart, pancreas, liver, skin, bone tissue, severed extremities or skeletal muscle.

54. Use of a polypeptide or a polypeptide composition according to any of claims 1-24 in connection with heart surgery.

30   55. Use of a polypeptide or a polypeptide composition according to any of claims 1-24 in connection with exposure to oxygen radical-producing toxic substances, or in connection with exposure to car-cinogens.

56. Use of a polypeptide or a polypeptide composition according to any of claims 1-24 in connection with pathological conditions involving superoxide radical-induced degradation of endothelium-derived vessel relaxant factor.

5   57. Use of a polypeptide or a polypeptide composition according to any of claims 1-24 in connection with cornea transplantations, preservation of lens and cornea, and other ophthalmic surgical procedures.

58. Use according to any of claims 51-57 in which the polypeptide or

10   a polypeptide composition according to any of claims 1-24 has a half-life in blood vessels of at least 4 hours.

60. A method of preventing or treating a disease or disorder connected with the presence or formation of superoxide radicals, comprising administering, to a patient in need of such treatment, a

15   therapeutically or prophylactically effective amount of a polypeptide or a polypeptide composition according to any of claims 1-24.

61. A method according to claim 60, in which the disease or disorder is selected from conditions involving ischemia followed by reperfusion, e.g. infarctions such as heart, kidney, brain or intestine

20   infarctions, inflammatory diseases such as rheumatoid arthritis, pancreatitis, in particular acute pancreatitis, pyelonephritis and other types of nephritis, and hepatitis, neuritis, uveitis, cystitis, peyronies disease, autoimmune diseases, insulin-dependent diabetes mellitus, disseminated intravascular coagulation, fatty embolism,

25   haemorrhagic shock, endotoxin-induced shock, septicemia, severe viral infections, adult respiratory distress, infantile respiratory distress, brain haemorrhages in neonates, burns, preservation of lens and cornea in the context of transplantation, adverse effects of ionizing radiation, and carcinogenesis, and adverse effects of toxins

30   such as alloxan, paraquat, and some cytostatic compounds.

62. A method of preventing or treating damage caused by ischemia followed by reperfusion in connection with the transplantation of

organs such as kidney, lung, pancreas, liver, skin, bone tissue, severed extremities or skeletal muscle, or in connection with heart surgery, comprising administering a therapeutically or prophylactically effective amount of a polypeptide or a polypeptide composition

5   according to any of claims 1-24 before, during or after surgery.

63. A method according to claim 60 or 62, in which a polypeptide or a polypeptide composition according to any of claims 1-24 is administered in a dosage of about 15-600 mg/day.

64. A method according to claim 60 or 62, which further comprises the

10   administration of heparin, heparan sulphate, other sulphated glucosaminoglycanes, dextran sulphate, or other strongly negatively charged compounds.

65. A method according to claim 60 or 62, which further comprises administering catalase or another antioxidant cooperating with a

15   polypeptide or a polypeptide composition according to any of claims 1-24 to reduce the toxic effects of oxygen reduction products.

66. A pharmaceutical composition which comprises a polypeptide or a polypeptide composition as claimed in any of claims 1-24 together with a pharmaceutically acceptable excipient, diluent or vehicle.

20   67. A pharmaceutical composition according to claim 66, which further comprises heparin, heparan sulphate, other sulphated glucosaminoglycanes, dextran sulphate, or other strongly negatively charged compounds, or a thrombolytic agent such as urokinase, streptokinase, tissue plasminogen activator or variants and mutants of these fac-

25   tors.

68. A pharmaceutical composition according to claim 66 or 67 which further comprises catalase, or another antioxidant cooperating with the polypeptide or polypeptide composition as claim in any of claims 1-24 to reduce the effects of oxygen reduction products.

69. A monoclonal antibody raised against or directed substantially only against a polypeptide or polypeptide composition as claimed in any of claims 1-13 or an immunological determinant thereof.

70. A monoclonal antibody according to claim 69 which is in substan-
5   tially pure form.

Fig. 1A   1/27

```
+1                                      10
TrpThrGlyGluAspSerAlaGluProAsnSerAspSerAlaGluTrpIleArgAsp
TGGACGGGCGAGGACTCGGCGGAGCCCAACTCTGACTCGGCGGAGTGGATCCGAGAC
                                                          180


   20
MetTyrAlaLysValThrGluIleTrpGlnGluValMetGlnArgArgAspAspAspGly
ATGTACGCCAAGGTCACGGAGATCTGGCAGGAGGTCATGCAGCGGCGGGACGACGACGGC
                                                          240

   40                            50
ThrLeuHisAlaAlaCysGlnValGlnProSerAlaThrLeuAspAlaAlaGlnProArg
ACGCTCCACGCCGCCTGCCAGGTGCAGCCGTCGGCCACGCTGGACGCCGCGCAGCCCCGG
                                                          300

   60                            70
ValThrGlyValValLeuPheArgGlnLeuAlaProArgAlaLysLeuAspAlaPhePhe
GTGACCGGCGTCGTCCTCTTCCGGCAGCTTGCGCCCCGCGCCAAGCTCGACGCCTTCTTC
                                                          360

   80                            90
AlaLeuGluGlyPheProThrGluProAsnSerSerSerArgAlaIleHisValHisGln
GCCCTGGAGGGCTTCCCGACCGAGCCGAACAGCTCCAGCCGCGCCATCCACGTGCACCAG
                                                          400

100                            110
PheGlyAspLeuSerGlnGlyCysGluSerThrGlyProHisTyrAsnProLeuAlaVal
TTCGGGGACCTGAGCCAGGGCTGCGAGTCCACCGGGCCCCACTACAACCCGCTGGCCGTG
                                                          480

120                            130
ProHisProGlnHisProGlyAspPheGlyAsnPheAlaValArgAspGlySerLeuTrp
CCGCACCCGCAGCACCCGGGCGACTTCGGCAACTTCGCGGTCCGCGACGGCAGCCTCTGG
                                                          540

140                            150
ArgTyrArgAlaGlyLeuAlaAlaSerLeuAlaGlyProHisSerIleValGlyArgAla
AGGTACCGCGCCGGCCTGGCCGCCTCGCTCGCGGGCCCGCACTCCATCGTGGGCCGGGCC
                                                          600

160                            170
ValValValHisAlaGlyGluAspAspLeuGlyArgGlyGlyAsnGlnAlaSerValGlu
GTGGTCGTCCACGCTGGCGAGGACGACCTGGGCCGCGGCGGCAACCAGGCCAGCGTGGAG
                                                          660

180                            190
AsnGlyAsnAlaGlyArgArgLeuAlaCysCysValValGlyValCysGlyProGlyLeu
AACGGGAACGCGGGCCGGCGGCTGGCCTGCTGCGTGGTGGGCGTGTGCGGGCCCGGGCTC
                                                          720
```

```
                         T209       T213    T215 T216
                         ***        ***     *** ***
200
TrpGluArgGlnAlaArgGluHisSerGluArgLysLysArgArgArgGlu SerGluCys
TGGGAGCGCCAGGCGCGGGAGCACTCAGAGCGCAAGAAGCGGCGGCGCGAG AGCGAGTGC
                         TGA              TGA    TAG TGA   780
```

```
220
LysAlaAla***
AAGGCCGCCTGA
```

Fig. 1B

```
+1                                                      10
TrpThrGlyGluAspSerAlaGluProAsnSerAspSerAlaGluTrpIleArgAsp
TGGACGGGCGAGGACTCGGCGGAGCCCAACTCTGACTCGGCGGAGTGGATCCGAGAC
                                                      180

20
MetTyrAlaLysValThrGluIleTrpGlnGluValMetGlnArgArgAspAspAspGly
ATGTACGCCAAGGTCACGGAGATCTGGCAGGAGGTCATGCAGCGGCGGGACGACGACGGC
                                                      240

40                                    50
ThrLeuHisAlaAlaCysGlnValGlnProSerAlaThrLeuAspAlaAlaGlnProArg
ACGCTCCACGCCGCCTGCCAGGTGCAGCCGTCGGCCACGCTGGACGCCGCGCAGCCCCGG
                                                      300

60                                    70
ValThrGlyValValLeuPheArgGlnLeuAlaProArgAlaLysLeuAspAlaPhePhe
GTGACCGGCGTCGTCCTCTTCCGGCAGCTTGCGCCCCGCGCCAAGCTCGACGCCTTCTTC
                                                      360

                              G1
                              Gln 90
80                                    SerSerSerArgAlaIleHisValHisGln
AlaLeuGluGlyPheProThrGluProAsnSerSerSerArgAlaIleHisValHisGln
GCCCTGGAGGGCTTCCCGACCGAGCCGAACAGCTCCAGCCGCGCCATCCACGTGCACCAG
                              CAA                   400

100                                   110
PheGlyAspLeuSerGlnGlyCysGluSerThrGlyProHisTyrAsnProLeuAlaVal
TTCGGGGACCTGAGCCAGGGCTGCGAGTCCACCGGGCCCCACTACAACCCGCTGGCCGTG
                                                      480

120                                   130
ProHisProGlnHisProGlyAspPheGlyAsnPheAlaValArgAspGlySerLeuTrp
CCGCACCCGCAGCACCCGGGCGACTTCGGCAACTTCGCGGTCCGCGACGGCAGCCTCTGG
                                                      540

140                                   150
ArgTyrArgAlaGlyLeuAlaAlaSerLeuAlaGlyProHisSerIleValGlyArgAla
AGGTACCGCGCCGGCCTGGCCGCCTCGCTCGCGGGCCCGCACTCCATCGTGGGCCGGGCC
                                                      600

160                                   170
ValValValHisAlaGlyGluAspAspLeuGlyArgGlyGlyAsnGlnAlaSerValGlu
GTGGTCGTCCACGCTGGCGAGGACGACCTGGGCCGCGGCGGCAACCAGGCCAGCGTGGAG
                                                      660

180                                   190
AsnGlyAsnAlaGlyArgArgLeuAlaCysCysValValGlyValCysGlyProGlyLeu
AACGGGAACGCGGGCCGGCGGCTGGCCTGCTGCGTGGTGGGCGTGTGCGGGCCCGGGCTC
                                                      720

                                             SA216    SA219
                                             Ala      Ala
200                         210
TrpGluArgGlnAlaArgGluHisSerGluArgLysLysArgArgArgGluSerGluCys
TGGGAGCGCCAGGCGCGGGAGCACTCAGAGCGCAAGAAGCGGCGGCGCGAGAGCGAGTGC
                                             GCG      GCC

SA220
Ala
LysAlaAla***
AAGGCCGCCTGA
CCC
```

SUBSTITUTE SHEET

Fig. 1C   3/27

```
      +1                                              10
    TrpThrGlyGluAspSerAlaGluProAsnSerAspSerAlaGluTrpIleArgAsp
    TGGACGGGCGAGGACTCGGCGGAGCCCAACTCTGACTCGGCGGAGTGGATCCGAGAC
                                                             180


    20
MetTyrAlaLysValThrGluIleTrpGlnGluValMetGlnArgArgAspAspAspGly
ATGTACGCCAAGGTCACGGAGATCTGGCAGGAGGTCATGCAGCGGCGGGACGACGACGGC
                                                             240
    40                              50
ThrLeuHisAlaAlaCysGlnValGlnProSerAlaThrLeuAspAlaAlaGlnProArg
ACGCTCCACGCCGCCTGCCAGGTGCAGCCGTCGGCCACGCTGGACGCCGCGCAGCCCCGG
                                                             300
    60                              70
ValThrGlyValValLeuPheArgGlnLeuAlaProArgAlaLysLeuAspAlaPhePhe
GTGACCGGCGTCGTCCTCTTCCGGCAGCTTGCGCCCCGCGCCAAGCTCGACGCCTTCTTC
                                                             360
                            G1
                            Gln  90
    80                   ProAsnSerSerSerArgAlaIleHisValHisGln
AlaLeuGluGlyPheProThrGluProAsnSerSerSerArgAlaIleHisValHisGln
GCCCTGGAGGGCTTCCCGACCGAGCCGAACAGCTCCAGCCGCGCCATCCACGTGCACCAG
                            CAA                              400
    100                             110
PheGlyAspLeuSerGlnGlyCysGluSerThrGlyProHisTyrAsnProLeuAlaVal
TTCGGGGACCTGAGCCAGGGCTGCGAGTCCACCGGGCCCCACTACAACCCGCTGGCCGTG
                                                             480
    120                             130
ProHisProGlnHisProGlyAspPheGlyAsnPheAlaValArgAspGlySerLeuTrp
CCGCACCCGCAGCACCCGGGCGACTTCGGCAACTTCGCGGTCCGCGACGGCAGCCTCTGG
                                                             540
    140                             150
ArgTyrArgAlaGlyLeuAlaAlaSerLeuAlaGlyProHisSerIleValGlyArgAla
AGGTACCGCGCCGGCCTGGCCGCCTCGCTCGCGGGCCCGCACTCCATCGTGGGCCGGGCC
                                                             600
    160                             170
ValValValHisAlaGlyGluAspAspLeuGlyArgGlyGlyAsnGlnAlaSerValGlu
GTGGTCGTCCACGCTGGCGAGGACGACCTGGGCCGCGGCGGCAACCAGGCCAGCGTGGAG
                                                             660
    180                             190
AsnGlyAsnAlaGlyArgArgLeuAlaCysCysValValGlyValCysGlyProGlyLeu
AACGGGAACGCGGGCCGGCGGCTGGCCTGCTGCGTGGTGGGCGTGTGCGGGCCCGGGCTC
                                                             720
                                              SAT216
                                              Ala***
    200                             210     ArgArgArgGluSerGluCys
TrpGluArgGlnAlaArgGluHisSerGluArgLysLysArgArgArgGluSerGluCys
TGGGAGCGCCAGGCGCGGGAGCACTCAGAGCGCAAGAAGCGGCGGCGCGAGAGCGAGTGC
                                              GCGTGA    780
    220
LysAlaAla***
AAGGCCGCCTGA
```

SUBSTITUTE SHEET

Fig. 2   4/27

Fig. 3   5/27

```
                              ***
T216       5'-GCGGCGCGAGTGAGAGTGCAAGG-3'
              ||||||||||| | ||||||||||
Template   3'-CGCCGCGCTCTCGCTCACGTTCC
                 |                    |
                762                  784


                              ***
T215       5'-GCGGCGGCGCTAGAGCGAGTG-3'
              |||||||||| ||||||||||
Template   3'-CGCCGCCGCGCTCTCGCTCAC-5'
                 |                  |
                759                779


                              ***
T213       5'-CAAGAAGCGGTGACGCGAGAGCG-3'
              |||||||||| | ||||||||||
Template   3'-GTTCTTCGCCGCCGCGCTCTCGC-5'
                 |                    |
                753                  775


                              ***
T209       5'-GCACTCAGAGTGAAAGAAGCGGC-3'
              |||||||||| | ||||||||||
Template   3'-CGTGAGTCTCGCGTTCTTCGCCG-5'
                 |                    |
                741                  763
```

SUBSTITUTE SHEET

Fig. 3 cont. 6/27

```
                           ***
SA216      5'-CGGCGGCGCGCGAGCGAGTGC-3
              ||||||||||| |||||||||
Template   3'-GCCGCCGCGCTCTCGCTCACG-5'
              |                   |
             760                 780
```

```
                           ***
SA219      5'-CGAGAGCGAGGCCAAGGCCGCCT-3'
              |||||||||| |||||||||||
Template   3'-GCTCTCGCTCACGTTCCGGCGGA-5'
              |                     |
             768                   790
```

```
                           ***
SA220      5'-GAGCGAGTGCGCGGCCGCCTGA-3'
              ||||||||| ||||||||||
Template   3'-CTCGCTCACGTTCCGGCGGACT-5'
              |                   |
             771                 792
```

```
                         ******
SAT216     5'-CGGCGGCGCGCGTGAGAGTGC-3
              |||||||||| | | ||||||
Template   3'-GCCGCCGCGCTCTCGCTCACG-5'
              |                   |
             760                 780
                           ***
```

```
G1         5'-GACCGAGCCGCAAAGCTCCAGCC-3'
              |||||||||| | |||||||||
Template   3'-CTGGCTCGGCTTGTCGAGGTCGG-5'
              |                     |
             378                   400
```
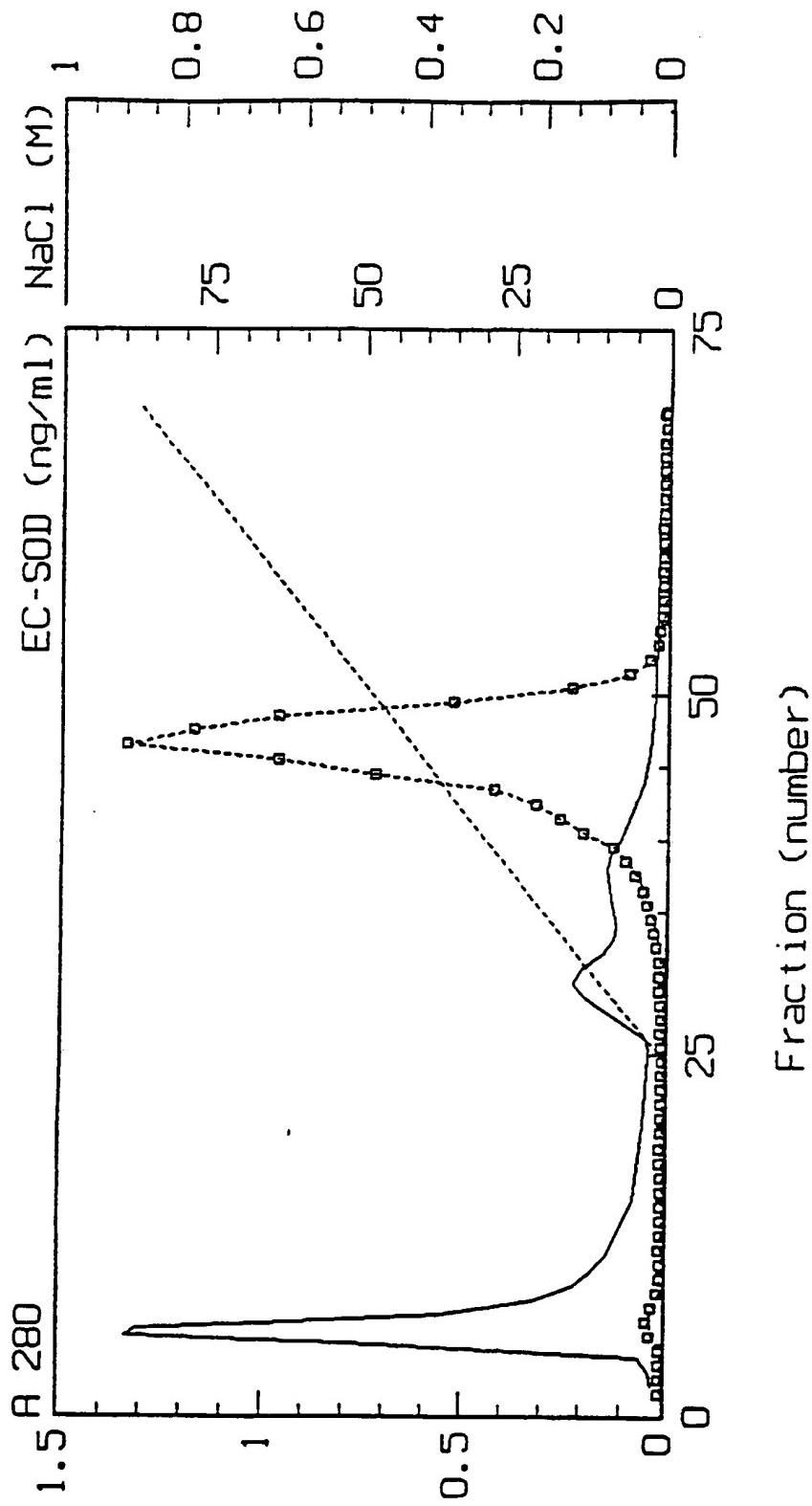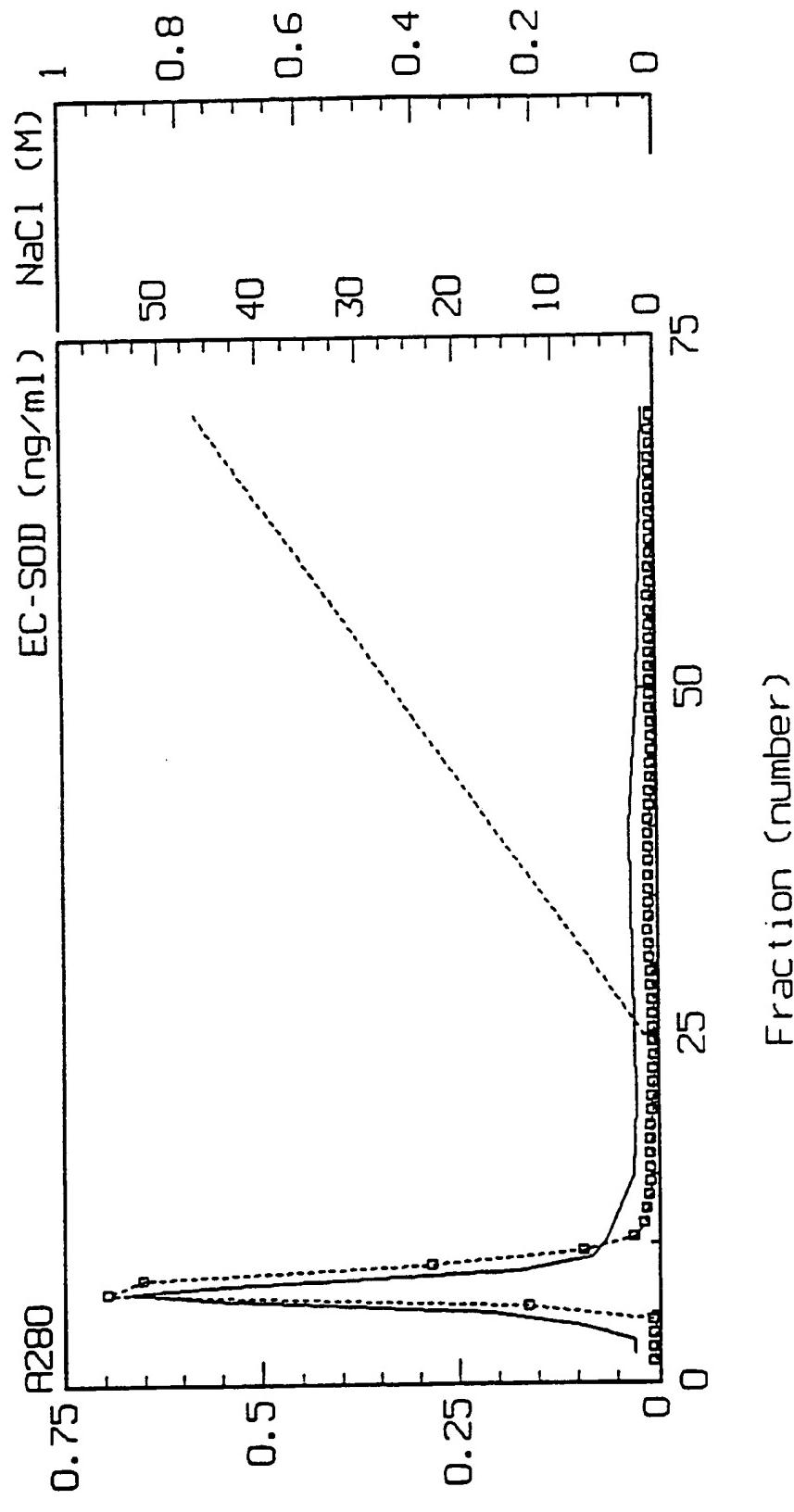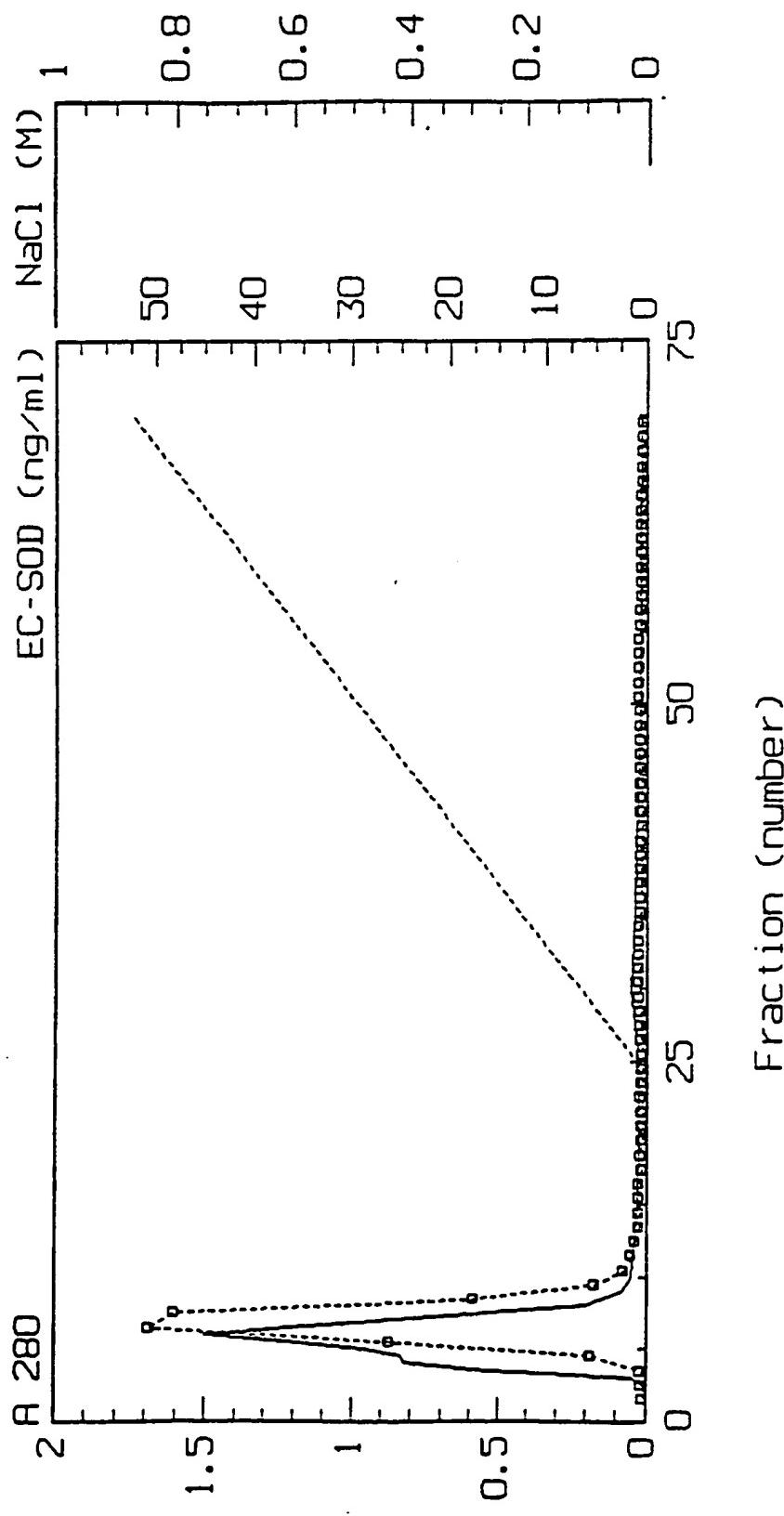
Fig. 4   7/27

Fig. 5

Fig. 6   9/27

Fig. 7
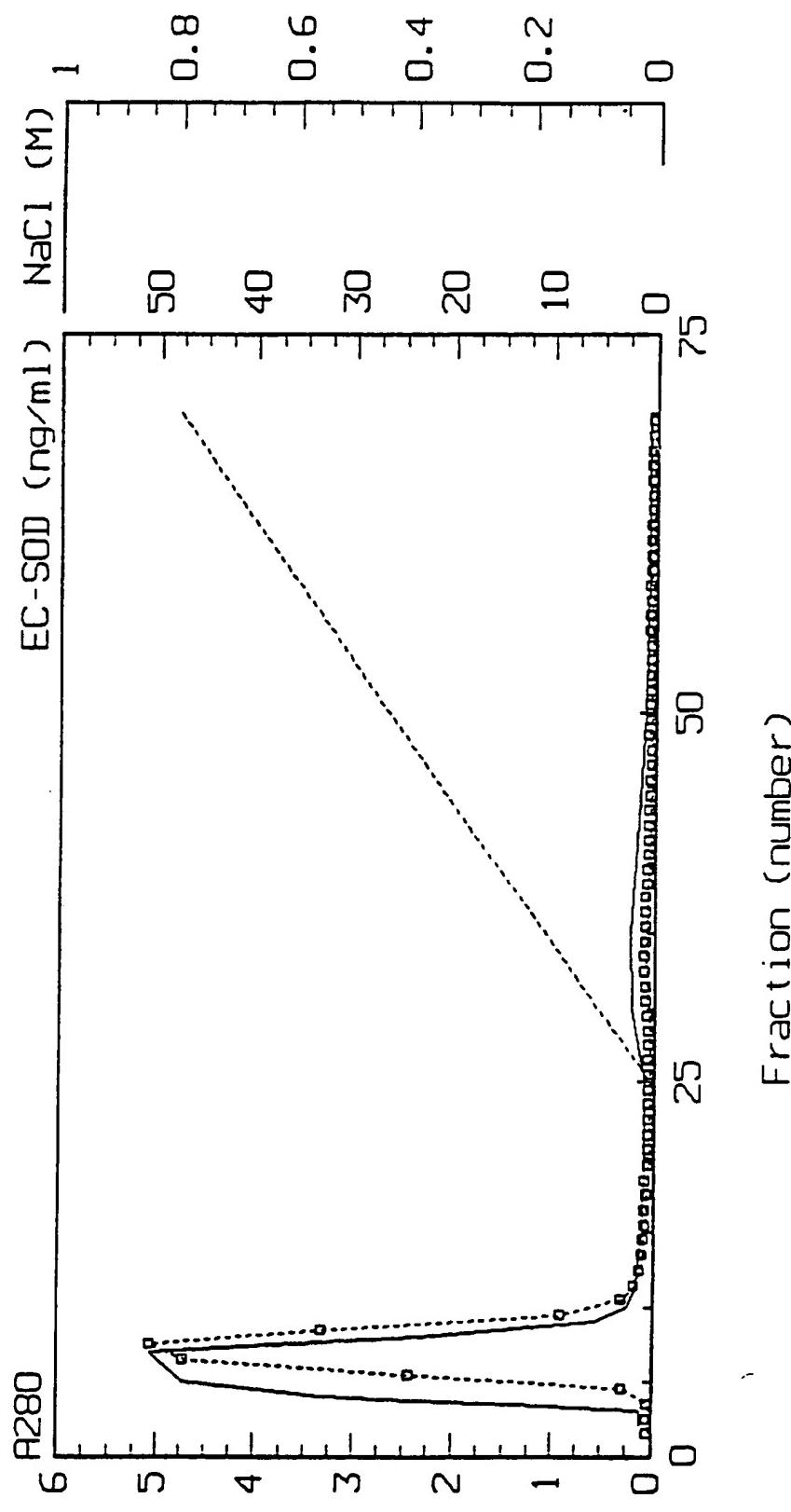
Fig. 8A   11/27
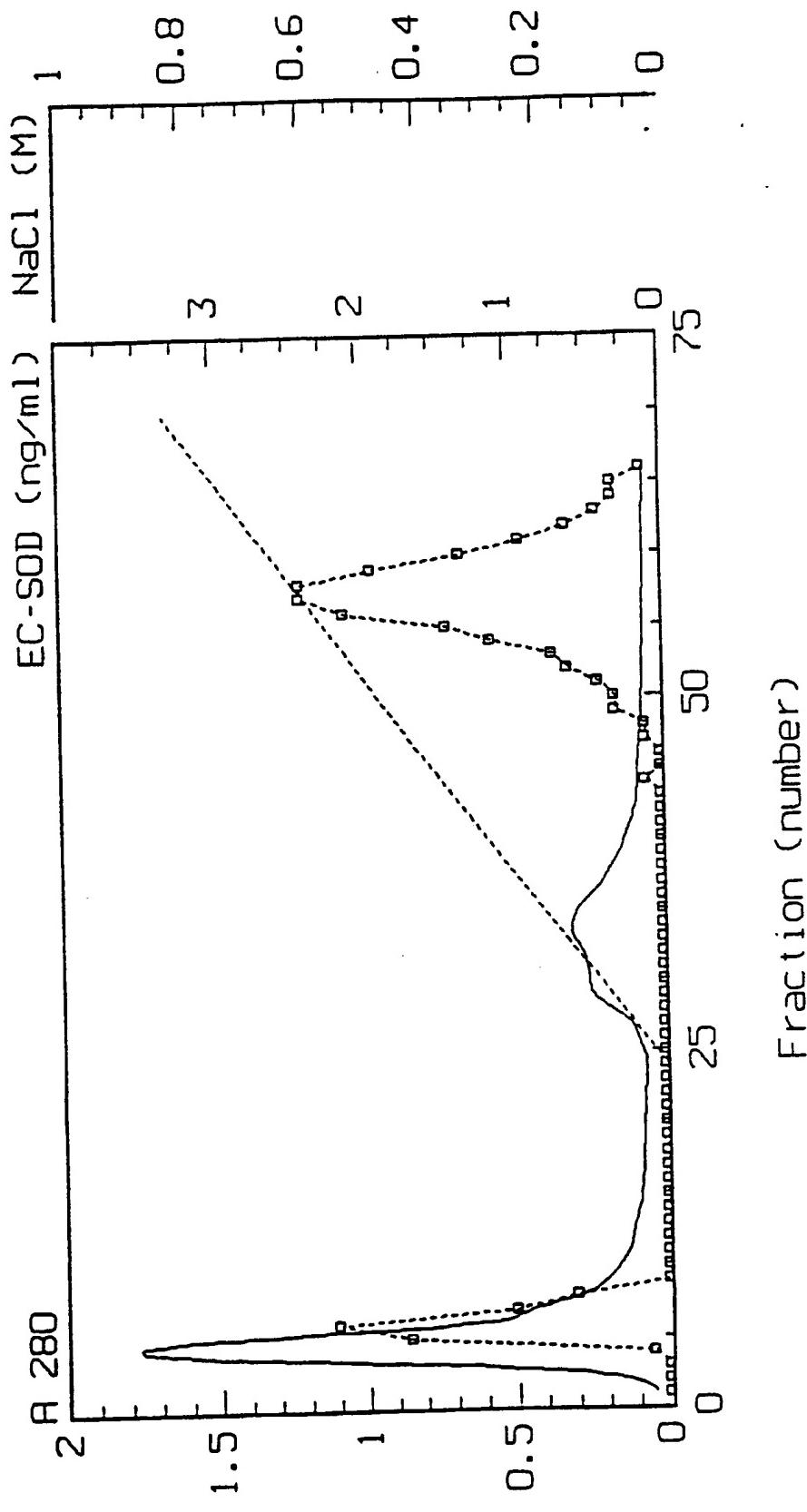
Fig. 8B   12/27
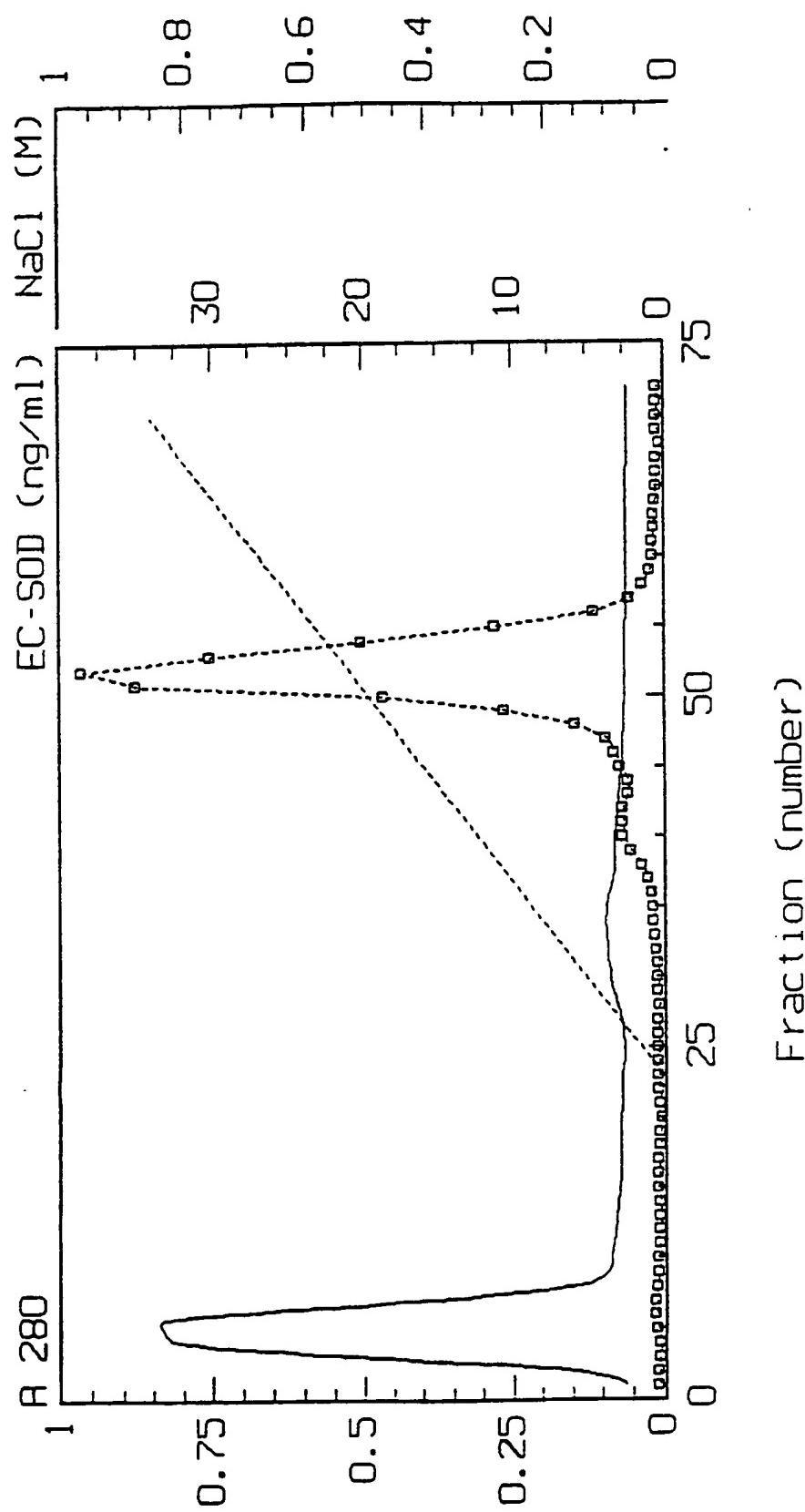
Fig. 8C   13/27
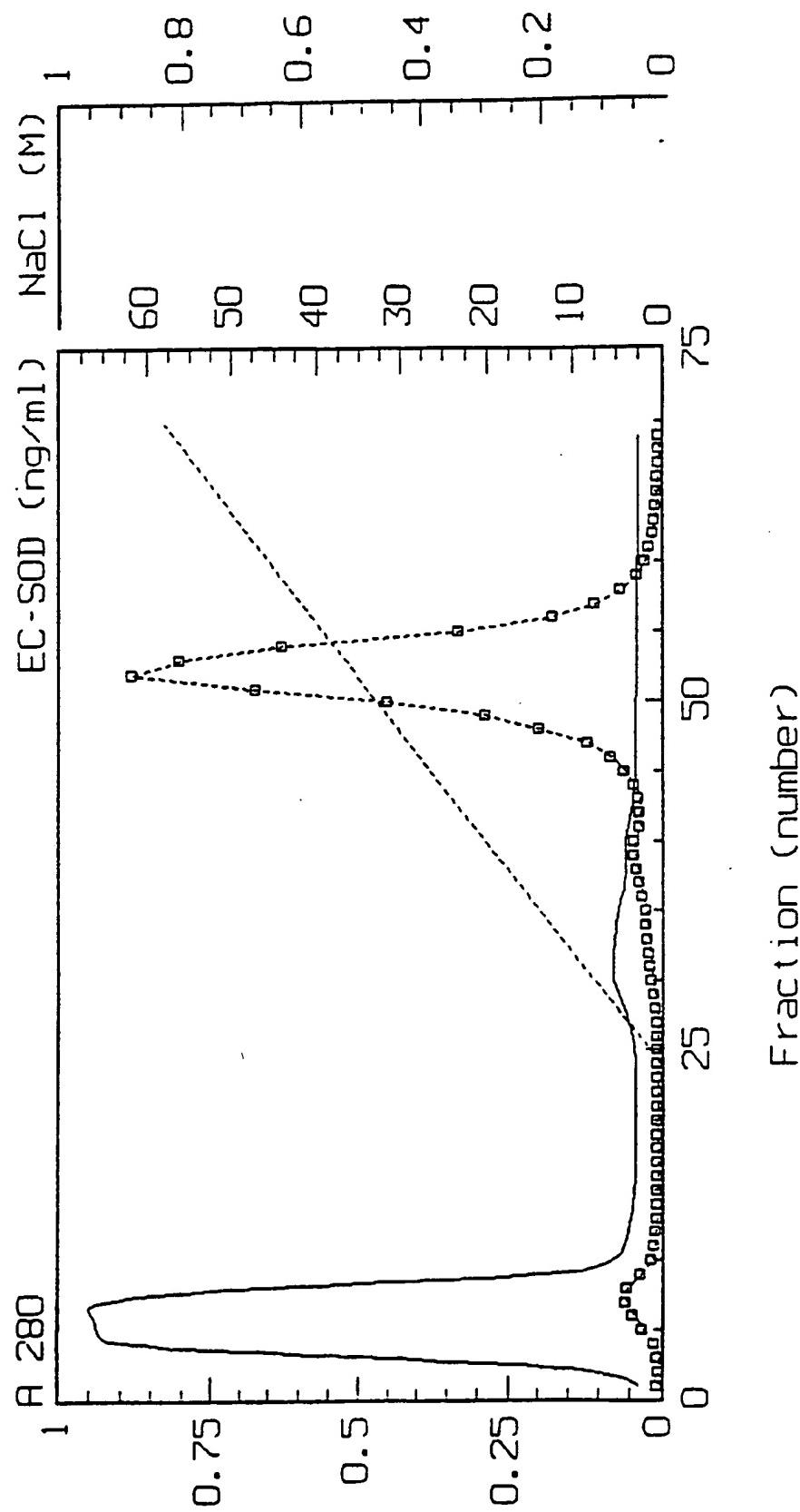
Fig. 8D　　　14/27
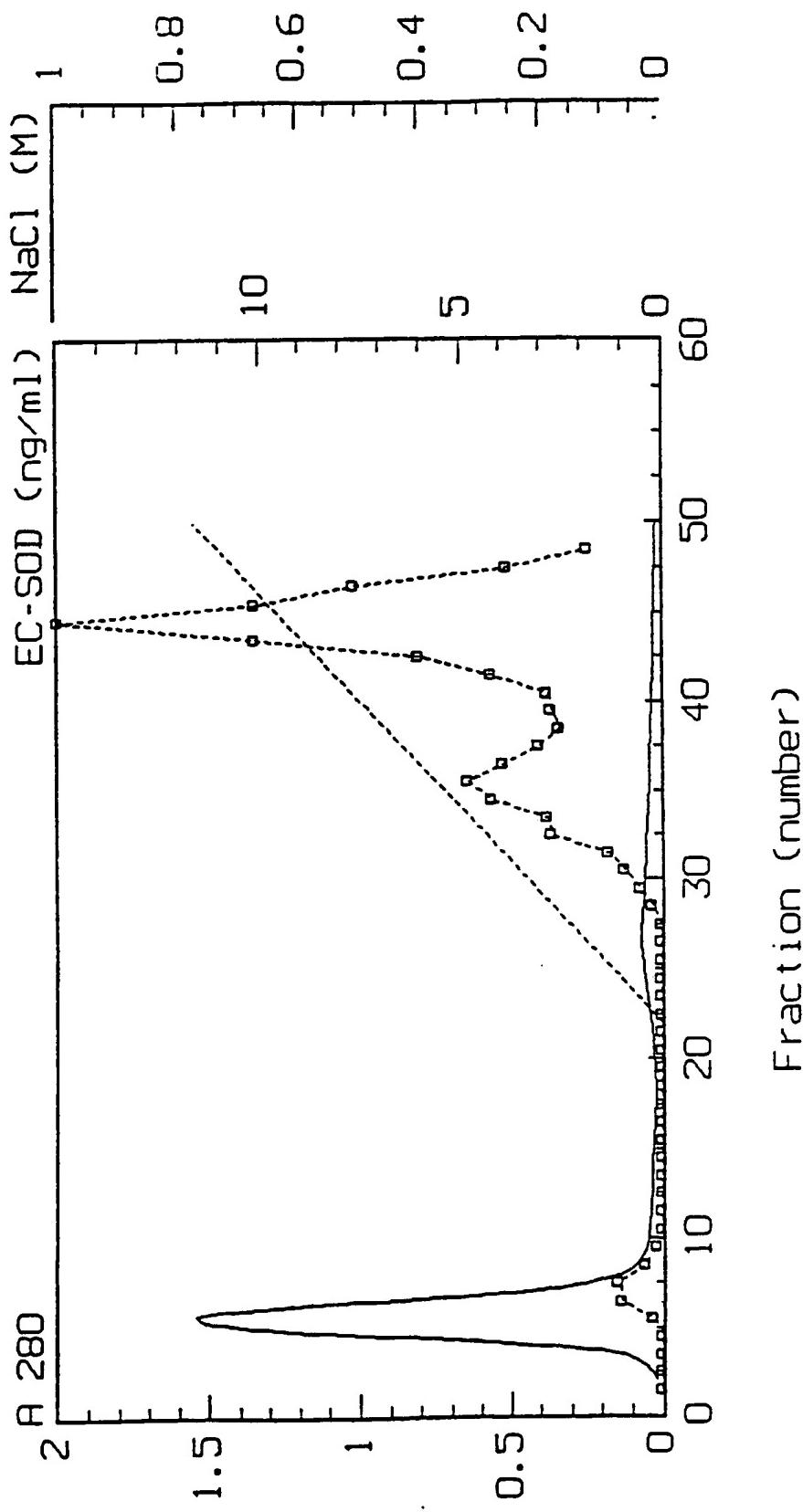
Fig. 8 E 15/27
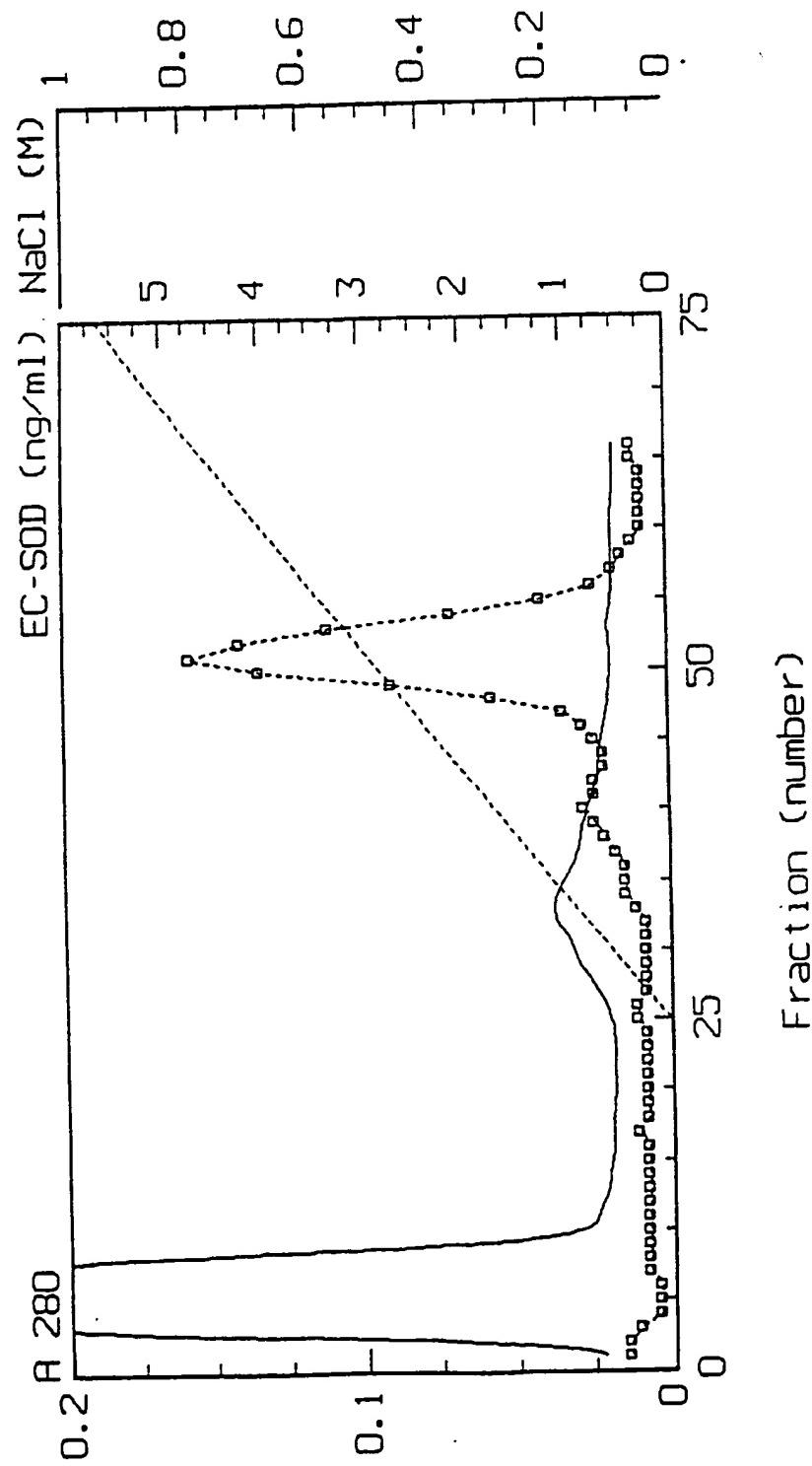
Fig. 9   16/27
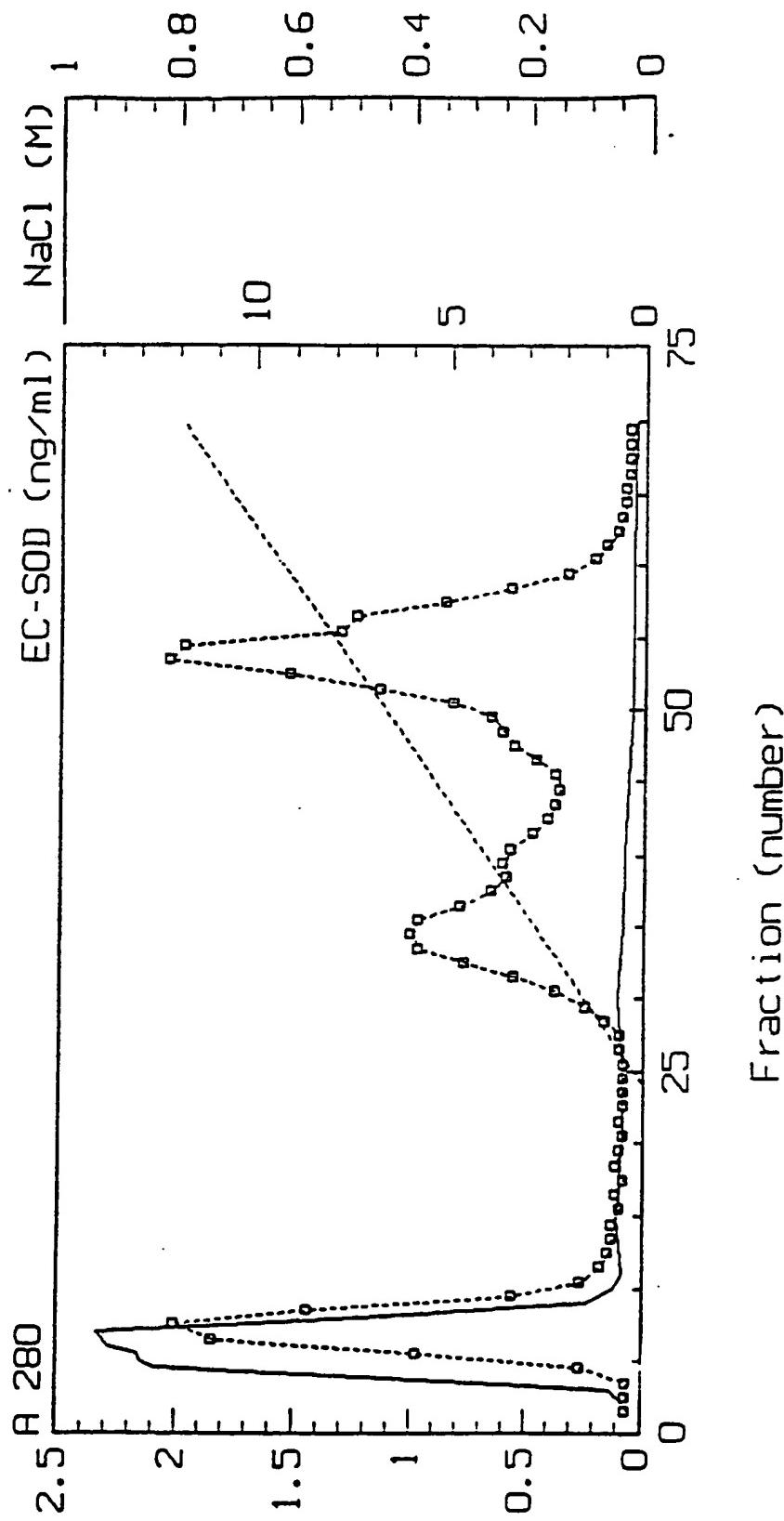
Fig. 10   17/27
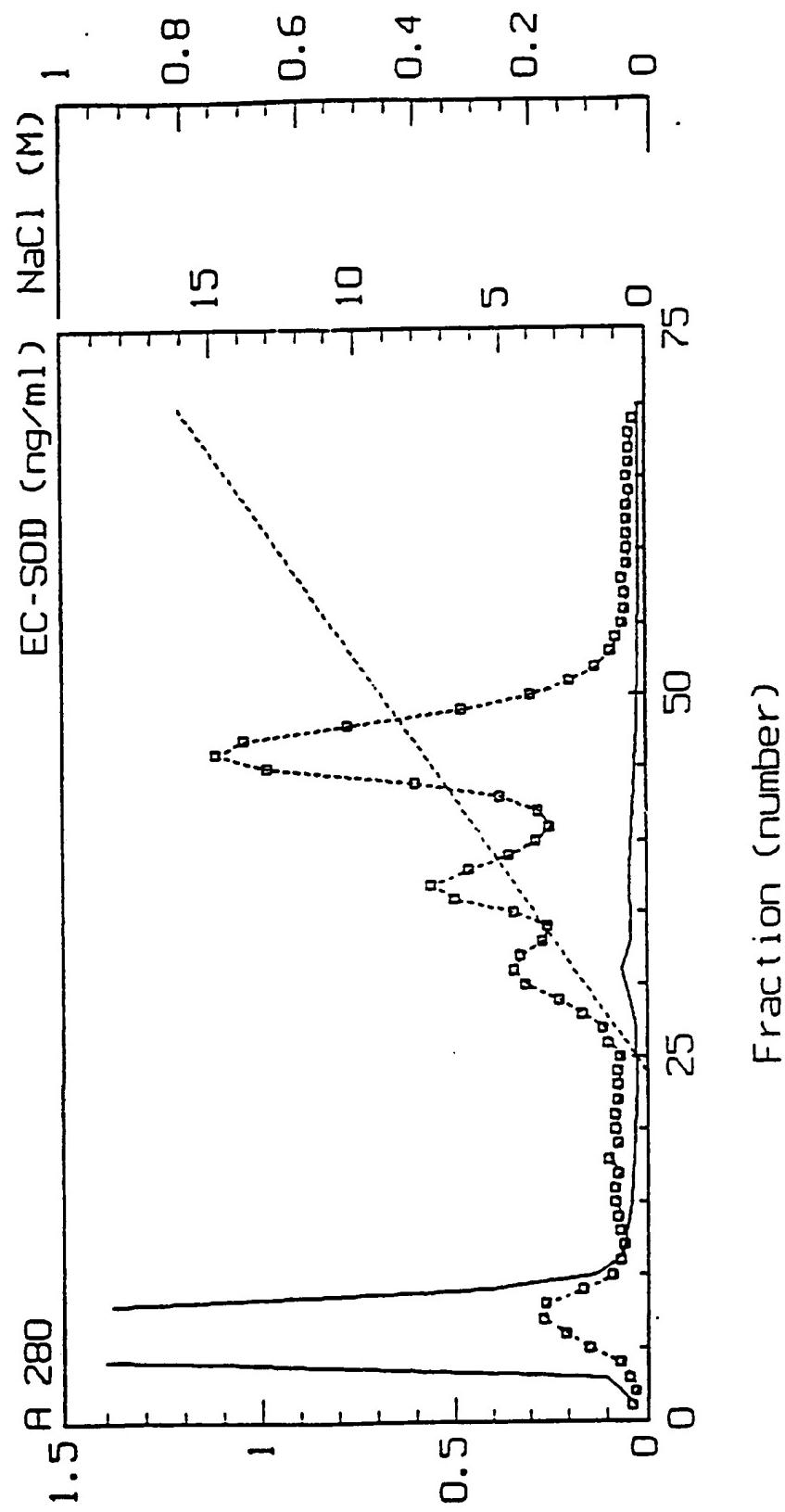
Fig. 11   18/27
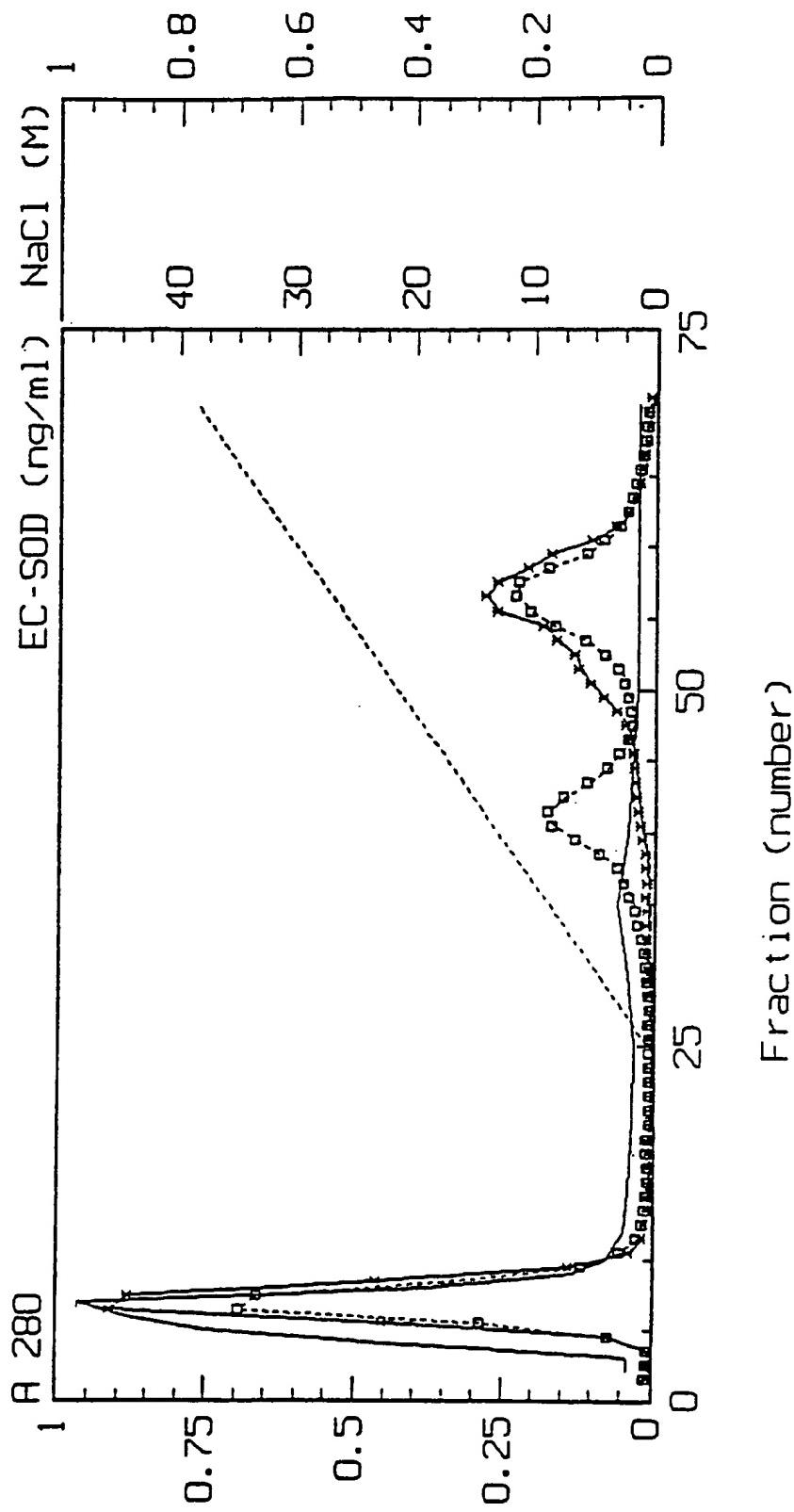
Fig. 12   19/27
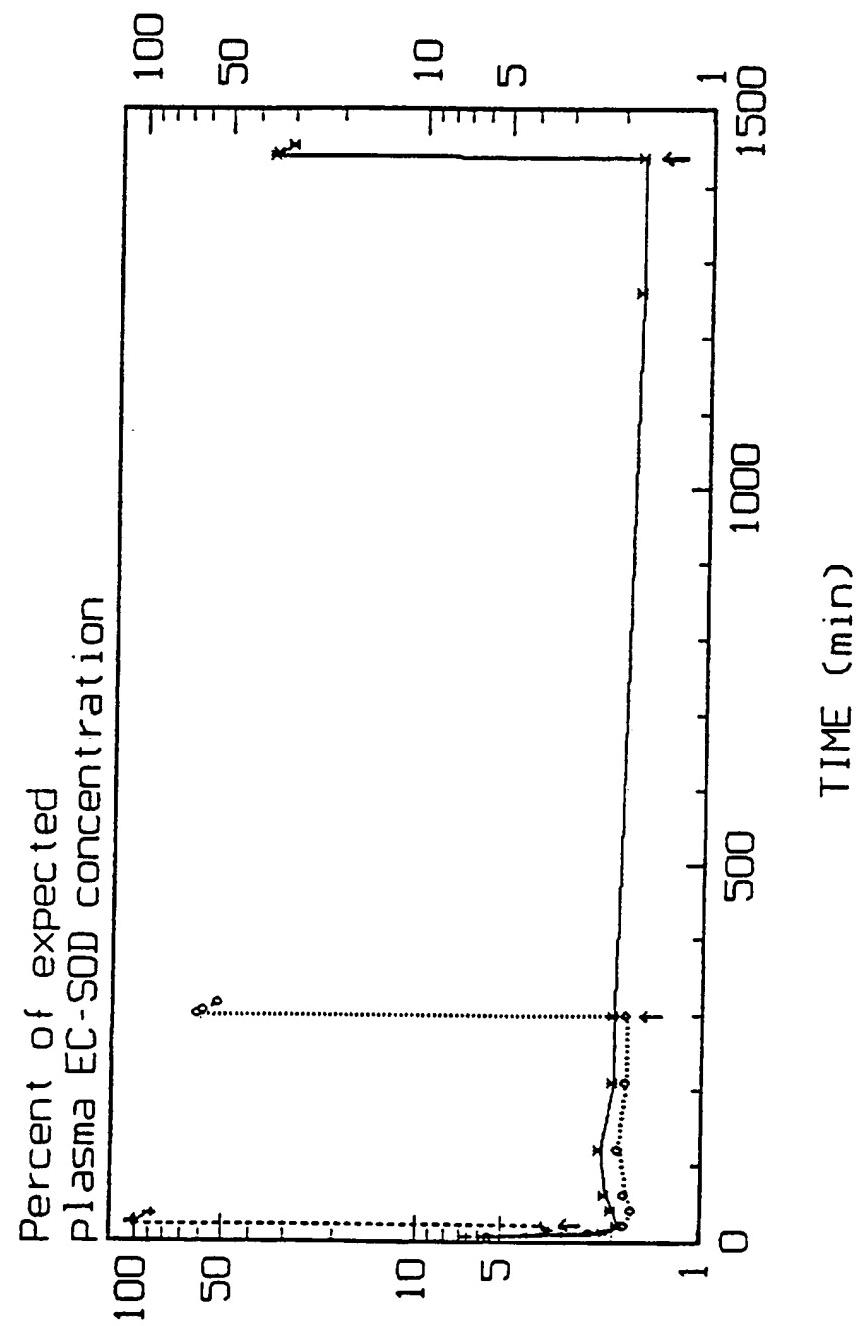
Fig. 13   20/27
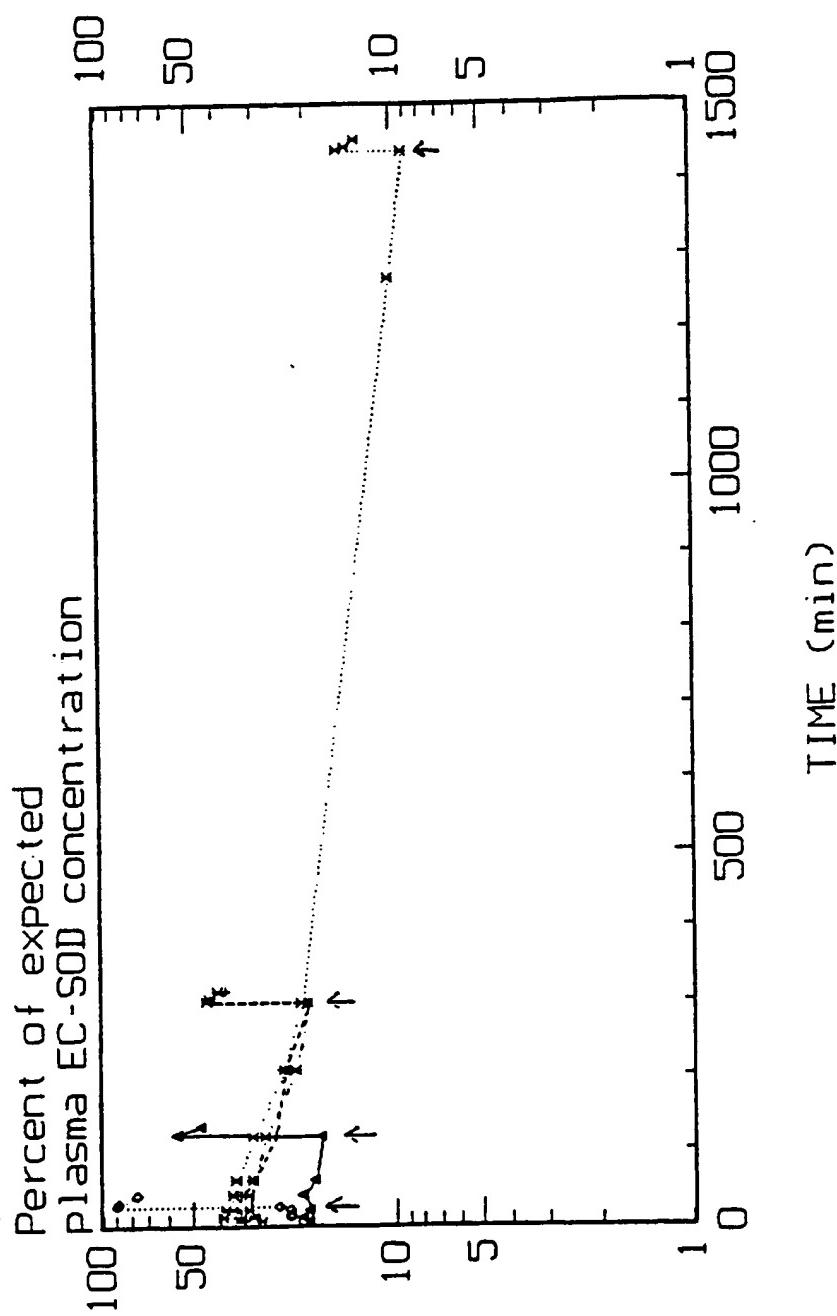
Fig. 14   21/27
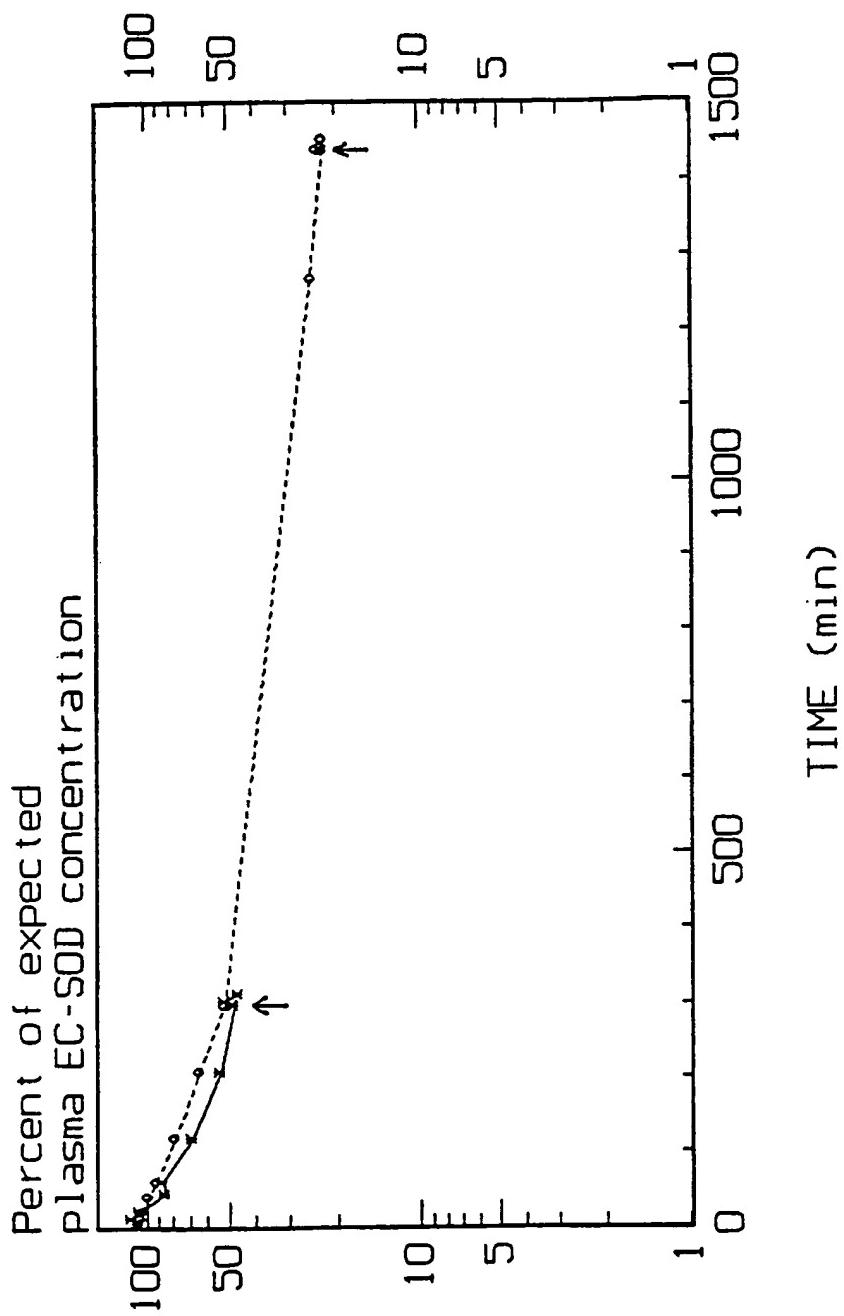
Fig. 15   22/27
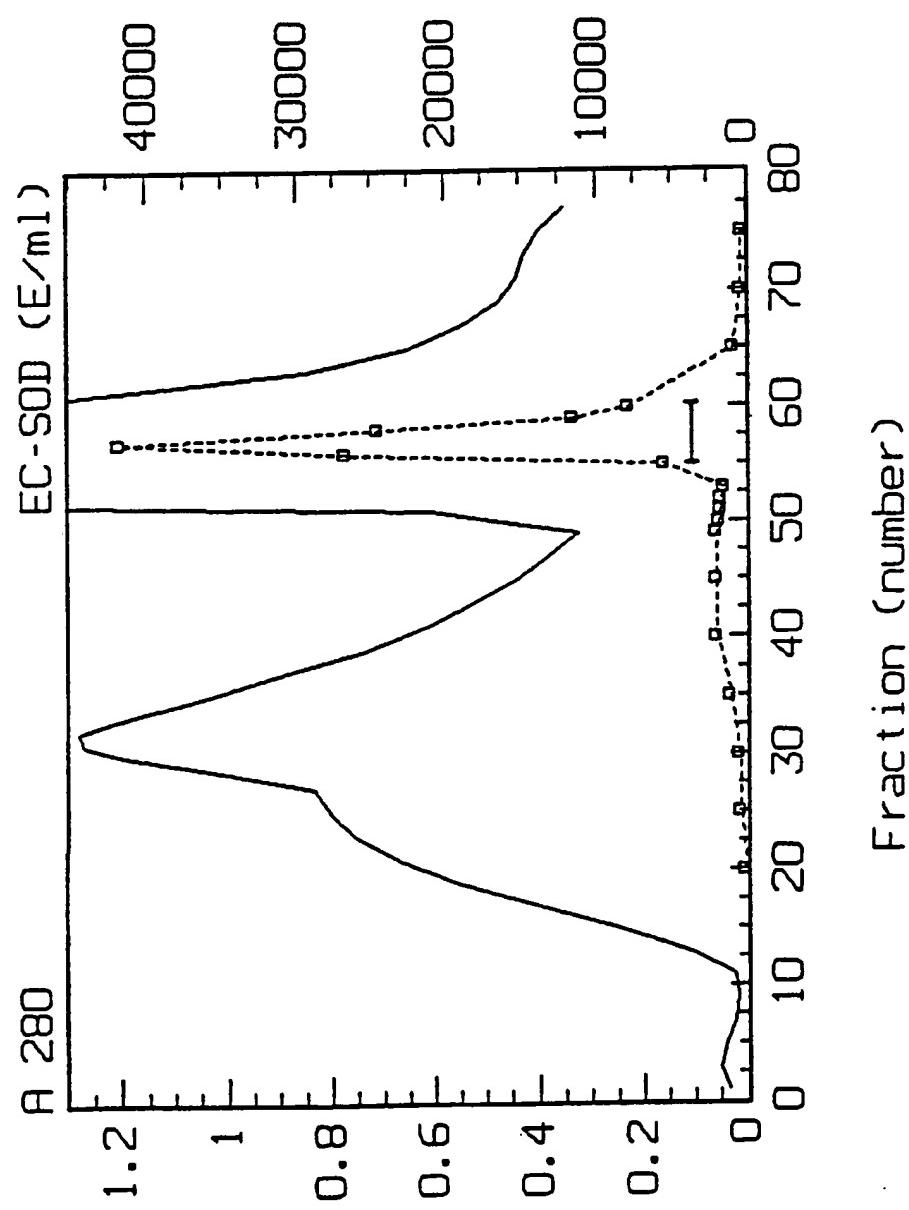
Fig. 16   23/27
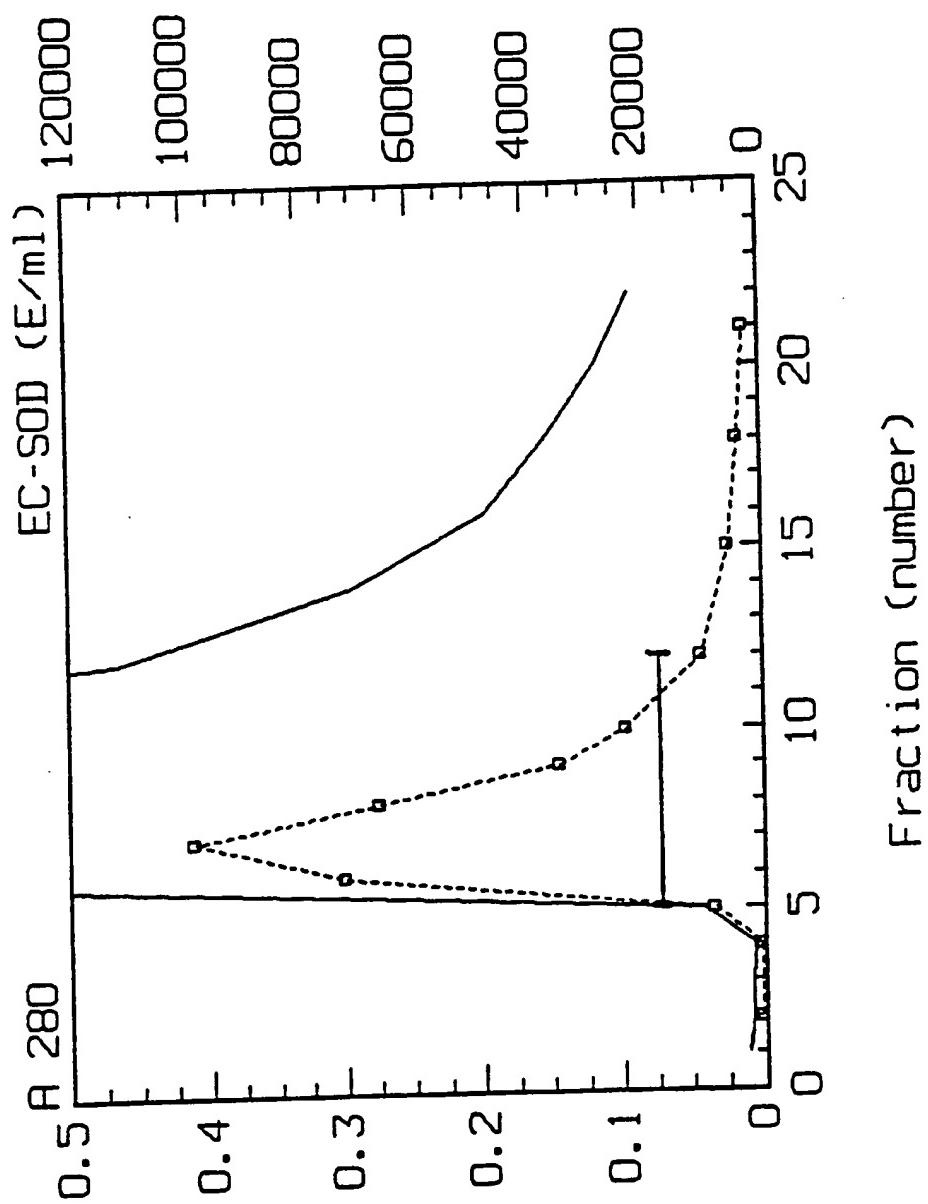
Fig. 17   24/27
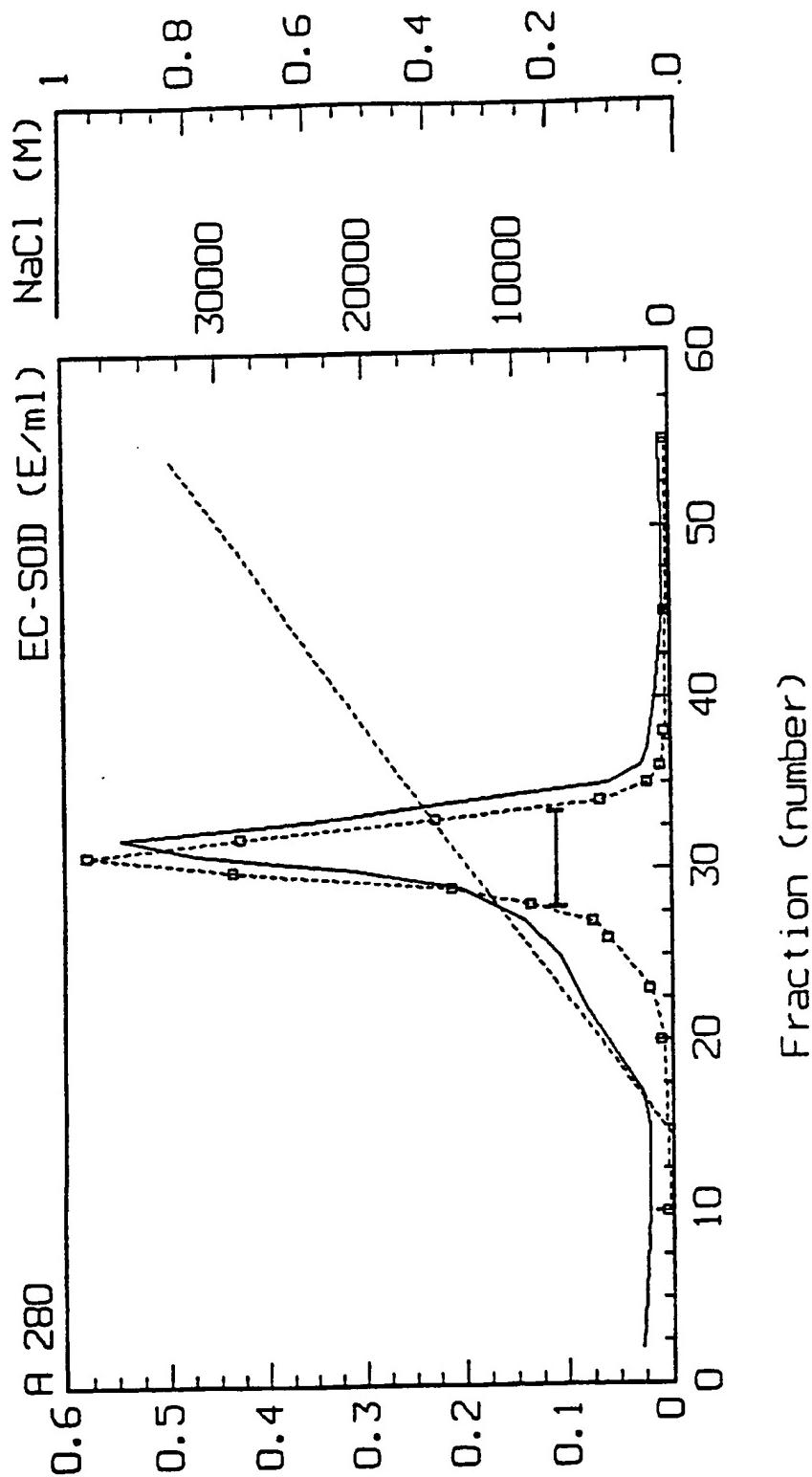
D   C   B   A

Fig. 18

A   B   C   D   E   F

Fig. 19

Fig. 20   27/27
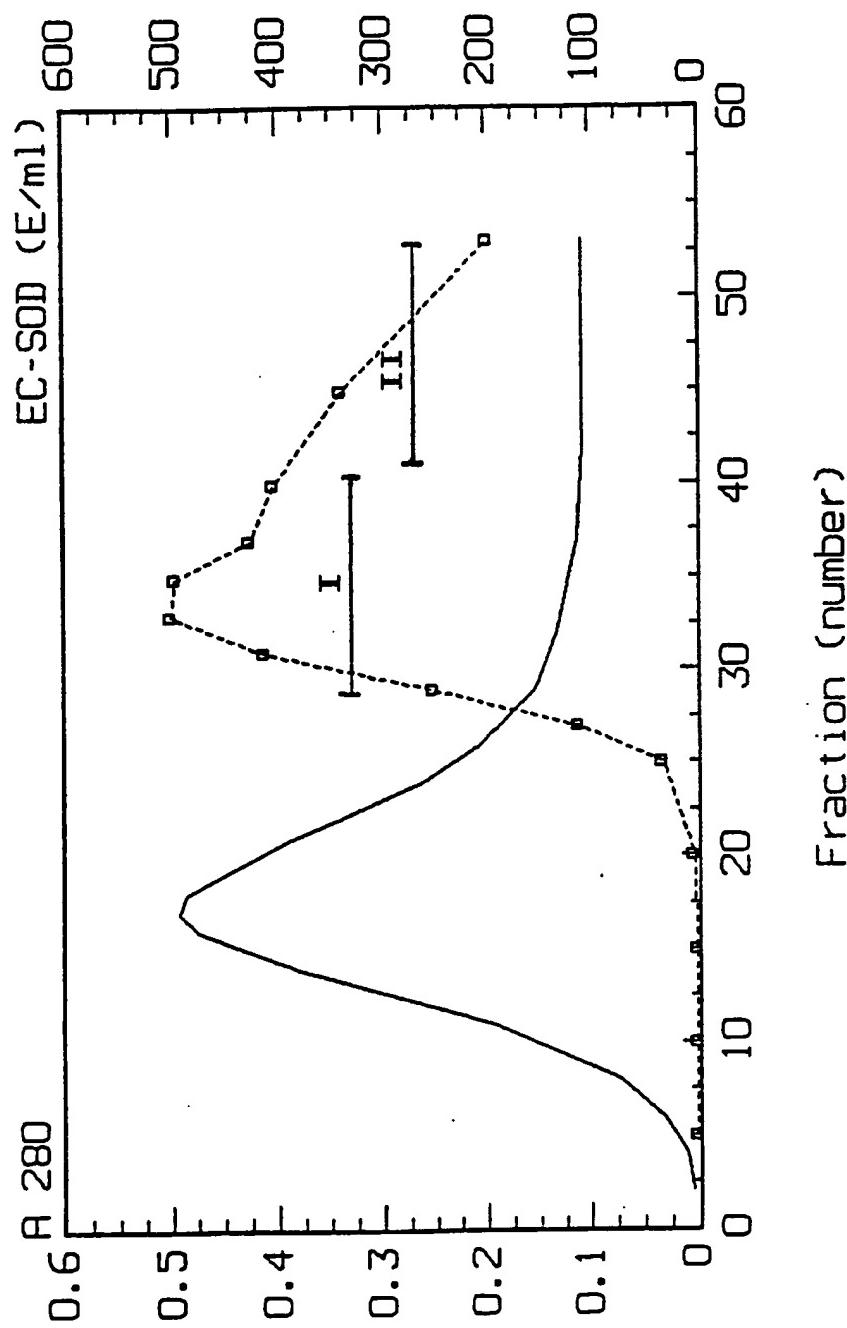
INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00238

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N, C 07 K, C 12 N

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 112 N; C 07 K; C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 9010694 (SUNTRY LIMITED JP/JP) 20 September 1990, see the whole document	1-6,26-30,32-34,42, 46-58, 66-70
X	Dialog Information Services, File 154, Medline 83-90. Dialog accession no. 89255306, Adachi T et al: "Interactions between human extracellular superoxide dismutase C and sulfated polysaccharides", J Biol Chem(UNITED STATES) May 25 1989, 264 (15) p 8537-41	1,26-30, 46-50
X	Dialog Information Services, File 154, Medline 83-90. Dialog accession no. 87317647, Hjalmarsson K et al: "Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase", Proc Natl Acad Sci U S A Sep 1987, 84 (18) p 6340-4	1,26-30, 46-50

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th December 1990	1990 -12- 18

International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)
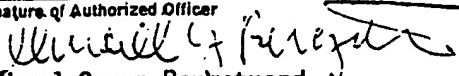
III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with Indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0275202 (CHIRON CORPORATION) 20 July 1988, see the whole document --	1-59
A	WO, A1, 8701387 (SYN-TEK AB) 12 March 1987, see the whole document --	1-59,66-70
A	EP, A2, 0213628 (YEDA RESEARCH AND DEVELOPMENT COMPANY, LTD) 11 March 1987, see the whole document --	1-59,66-70
A	EP, A1, 0138111 (CHIRON CORPORATION) 24 April 1985, see the whole document --	1-59,66-70

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on   90-11-28
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WO-A1- 8701387	87-03-12	AU-B-	598756	90-07-05
		AU-D-	6370886	87-03-24
		EP-A-	0236385	87-09-16
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		JP-A-	62115277	87-05-26
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